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PHYSIOLOGICAL REVIEWS

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PHYSIOLOGICAL REVIEWS

VOL. 26 APRIL, 1946 No. 2

SYMPATHOMIMETIC AMINES: THE RELATION OF STRUCTURE TO THEIR ACTION AND INACTIVATION

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We are dealing with a range of compounds which simulate the effects of sympathetic nerves not only with varying intensity but with varying precision. In some cases the points of chief interest in the action of a compound are those in which it differs from the action of adrenine. A term at once wider and more descriptive than "adrenine-like" seems needed to indicate the type of action common to these bases. We propose to call it "sympathomimetic," a term which indicates the relation of action to innervation by the sympathetic system, without involving any theoretical preconception as to the meaning of that relation or the precise mechanism of the action. Barger and Dale: J. Physiol. **41**: 19, 1910.

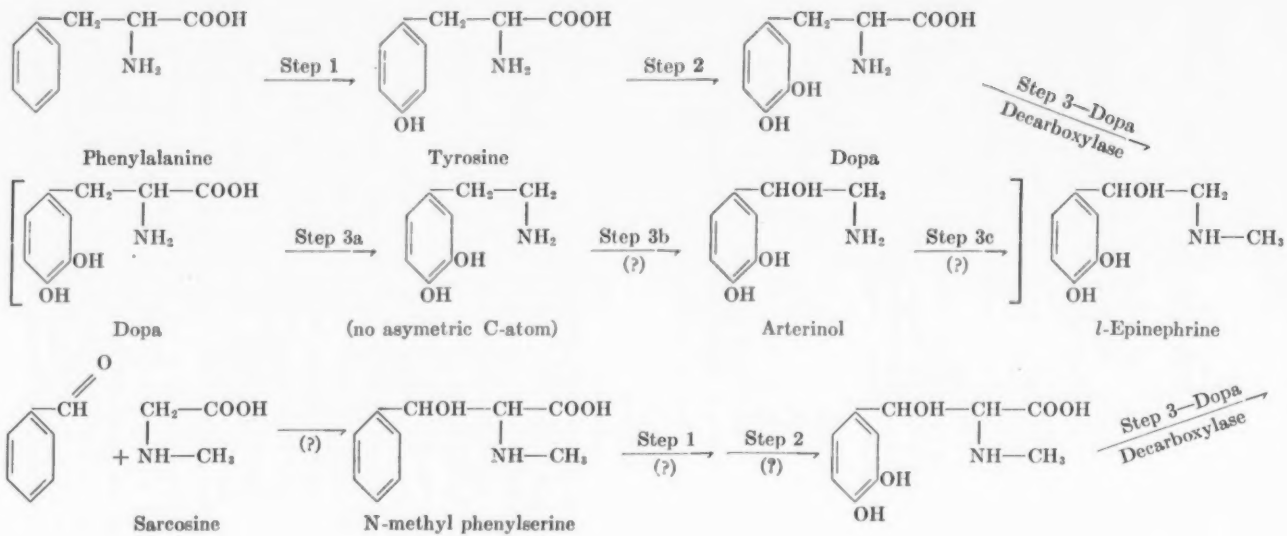
Reviews of the literature on sympathomimetic amines have appeared from time to time. Of these the ones by Chen and Schmidt (1) and Hartung (2) are comprehensive integrations of the work in this field through 1931. Since then there has appeared a number of briefer reviews (3), the most recent comprehensive treatment of the subject being the Symposium on Sympathomimetic Agents presented before the American Chemical Society in 1944 (4).

The purpose of this article is to review the formation of pressor amines in the body, and their relation to the pathogenesis and experimental alleviation of hypertension; to consider their pharmacodynamic effects on cellular or tissue metabolism as determining the mode of action of these agents; to review the relation of structure to the inactivation of sympathomimetic amines; and to present certain generalizations concerning the relation of structure of these compounds to their action and inactivation.

EPINEPHRINE FORMATION. The formation of epinephrine and other pressor amines in the body can be portrayed simply with pencil and paper although proof of the mode of synthesis of the hormone by mammalian tissues is quite incomplete. The equations used to illustrate the discussion of epinephrine synthesis are given in the accompanying diagram.

In our present discussion of these syntheses it is convenient to begin with phenylalanine since it has been shown to be an essential amino acid (5). Decarboxylation of this amino acid has been demonstrated to occur in the course of bacterial proteolysis with the formation of the pressor agent β-phenylethylamine (6). This reaction occurs in the digestive tract, the amine being absorbed and normally inactivated by the amine oxidase of the body (7).

The oxidation of phenylalanine to tyrosine (step 1) was shown to occur in the presence of iron and hydrogen peroxide (8). Recently the biological conversion of phenylalanine to tyrosine has received strong substantiation by the isolation of tyrosine containing deuterium from the bodies of rats whose diet has been supplemented with deuterophenylalanine (9). The decarboxylation of tyrosine


Phenylalanine
Step 1
Tyrosine
Step 2
Dopa
Step 3—Dopa Decarboxylase
Dopa
Step 3a
(no asymetric C-atom)
Step 3b
(?)
Arterinol
Step 3c
(?)
l-Epinephrine
Sarcosine
(?)
N-methyl phenylserine
Step 1
(?)
Step 2
(?)
Step 3—Dopa Decarboxylase

to yield the pressor agent tyramine occurs in the kidney (10) and pancreas (11) and is catalyzed by a decarboxylase (10b, 12). Bacteria have been shown to possess a similar carboxylase and very likely the decarboxylation of tyrosine in the intestine contributes considerably to that normally eliminated by the body (13).

The oxidation of tyrosine to 3,4 dihydroxyphenylalanine, "dopa," (step 2) can be initiated by enzymatic and nonenzymatic oxidative measures (14). The ascorbic-dehydroascorbic acid system is capable of oxidizing tyramine to the corresponding 3,4 dihydric derivative and stabilizing the reaction at that point (15) and presumably could do so for tyrosine. An analogous point is the stabilization of epinephrine by ascorbic acid in the adrenal gland (16). Also, Medes (17) reported the recovery of *l*-3,4 dihydroxyphenylalanine from urine when large amounts of tyrosine were administered to a patient diagnosed as having tyrosinosis.

The method for the decarboxylation of "dopa" in the mammalian organism rests on firmer structure. Holtz has demonstrated the presence of "dopa" decarboxylase in practically all animals capable of synthesizing epinephrine (18), and has shown it to function selectively in the decarboxylation of the *l*-amino acid, 3,4 dihydroxyphenylalanine (step 3). He proposed the theory that "dopa" decarboxylase may be an accessory enzyme important for the biological formation of epinephrine. His later diagrams show the decarboxylation of "dopa" to 3,4 dihydroxyphenylethylamine by the decarboxylase and another arrow pointing from that structure to one for epinephrine. According to the scheme of things as developed to this point there must be introduced into this agent, 3,4 dihydroxyphenylethylamine, both an aliphatic hydroxyl group and a methyl group on the amino nitrogen (steps 3b and 3c). Moreover, this feat must be accomplished under such stable conditions as not to wreck the very labile catechol nucleus. Heard and Raper have reported that by the action of tyrosinase on N-methyl "dopa" *in vitro* adrenalone was formed. However, perfusion of adrenalone through the adrenal gland did not result in its reduction to epinephrine (14b). Also, Vinet claimed that the adrenal medulla was capable of bringing about the oxidation and methylation of decarboxylated dihydroxyphenylalanine (19). An appreciation of the chemistry involved in such a transformation would make it seem logical to look about for other means whereby the end could be accomplished more subtly.

For example, it would be reasonable that starting with benzaldehyde or p-hydroxybenzaldehyde, or phenylglyoxylic acid as proposed by Rosenmund and Dornsaft (20), and N-methylglycine (sarcosine) the body could cause their condensation to form β-hydroxy-N-methylphenylalanine or its p-phenolic analogue. This general reaction requires only a low order of activation *in vitro* and is in effect an aldol condensation, which has been invoked to account for a number of biochemical reactions.

While this initial reaction and the subsequent ones (steps 1? and 2?) have no direct experimental substantiation, the resulting compound, N-methyl-phenylserine, should behave similarly to phenylalanine although possibly more

slowly with respect to enzymatic reactions in general, as outlined in the initial consideration of epinephrine synthesis. If this be so, the decarboxylation of *l*-N-methyl-3,4-dihydroxyphenylserine by Holtz' *l*-dopa decarboxylase would give rise immediately to the naturally occurring form of epinephrine (step 3). Starting with glycine instead of sarcosine one could explain similarly the formation of arterenol, which has been claimed to be the substance responsible for the mediation of adrenergic nerve impulses (21). Even if the latter course of reactions should take place leading to the synthesis of epinephrine it would not preclude the occurrence in the body of those initially discussed up to and including the formation of β-(3,4-dihydroxyphenyl)ethylamine. The fact that the subcutaneous injection of 3,4-dihydroxyphenylserine is reported not to cause an elevation of blood pressure in a dog (22) detracts no more from it as a precursor of epinephrine than the lack of pressor response when "dopa" is injected into a normal anesthetized animal (18a).

The recognition that the formation of various pressor agents having the basic β-phenylethylamine nucleus need not be direct steps in the formation of epinephrine, although such has been claimed to be the case (23), brings us to a consideration of these as causative agents in the pathogenesis of hypertension.

Hypertension. That naturally occurring sympathomimetic amines may be etiologic agents in hypertension is more a revival of interest than a new concept. In 1910 Ewins and Laidlaw commented in an introductory statement that the formation of tyramine from tyrosine in the intestine "has quite recently been regarded as playing a part in certain pathological states in which a high blood pressure is the most prominent symptom" (24). That same year Bain reported that tyramine was excreted in the urine of such patients but to a less extent than in patients having normal blood pressure (25), the implication being that it was retention of the amine that was responsible for the elevation of blood pressure. The urohypertensine of Abelous and Bardier (26) was in part isoamylamine, and recently Lockett (27) has isolated pressor agents from normal urine. Today the interest in the subject is the same, the emphasis on origin of these agents has changed.

Holtz (18) and Blaschko (28) both observed that "dopa" was decarboxylated to form an amine which possessed both hyperglycemic and pressor properties (18c). Following their initial investigations but preceding Holtz' later work Bing pointed out, as based on his own *in vivo* experimentation, the hypothetical relation of "dopa" to essential hypertension (29).

He demonstrated that when "dopa" was injected into a kidney deprived of its blood supply or partially ischemic, but otherwise intact in a cat, and allowed to incubate for a suitable period of time, the re-establishment of circulation through that kidney resulted in an elevation of blood pressure. This effect was interpreted as being due to the decarboxylation of "dopa" with the formation of the corresponding pressor amine in the ischemic kidney. Moreover, the anoxic condition of that organ precluded the deamination of the pressor amine, as interpreted by Bing. The explanation of these findings can be substantiated further by the citation of Holtz' report that he could demonstrate the formation

of β-(3,4-dihydroxyphenyl)ethylamine from "dopa" by the kidney or pancreas only when the organ was incubated with the amino acid in an atmosphere of nitrogen. This was necessary in order to block the oxidation of the resulting amine by amine oxidase (18a).

This acute experiment and its likely interpretation have led to the hypothesis that essential hypertension may be due to the decarboxylation of amino acid precursors of pressor amines in a damaged and ischemic kidney in which condition that organ is not capable of bringing about their deamination. For such a circumstance to obtain it must be supposed that 1, the predominant site of inactivation of these amines is the kidney; 2, that a rather severe state of renal ischemia is an integral part of the hypertension, and 3, that the principal mode of inactivation of the phenolic pressor amines is by deamination. Very likely none of these qualifications can be substantiated, except in an acute experiment where the amount of pressor amine liberated to the body on re-establishing circulation through the kidney is overwhelmingly greater than could be inactivated immediately by the body.

The fact that the kidney is neither the only or even the major source of amine oxidase has been demonstrated by Bhagvat, Blaschko and Richter who found it to be widely distributed in the body (30). The fact that β-phenyl-n-propylamine administered orally is inactivated by deamination and is not excreted as such unless a hepatotoxin, carbon tetrachloride, was previously administered, suggests the liver as a principal organ for deamination of that agent (31). It is difficult to believe that even under pathological conditions the rate of formation and liberation of such an agent by the kidney could exceed its rate of deamination by other organs. Brown and Maegraith could demonstrate no reduction of amine oxidase in the livers of hypertensive rats (32). Actually, there appears to be no amine oxidase in the kidney of the rat (33). Certainly in the rat, an animal widely used for experimental hypertension, faulty deamination of pressor amines cannot be considered as contributing to the hypertension produced by damage to the kidney.

It is doubtful whether a state of ischemia sufficient to abolish the functional capacity of an enzymatic oxidative system exists early in patients having essential hypertension, except in the rare instance of a lesion obstructing markedly the principal blood supply to the organ. The decrease in renal blood flow and elevation of filtration fraction occurs earliest in the disease and appears to precede a decrease in the functional capacity of the renal tubules (34). This order of things would at least make less likely impaired oxidation of decarboxylated "dopa" the initiating factor for the increased tonus of the vascular system.

The belief that phenolic pressor amines are inactivated in the body principally by deamination is not established as a certainty (35), although *in vitro* they can be shown to be deaminated rapidly by the enzyme. The nature of this problem more properly relates to the inactivation of sympathomimetic amines, which will be discussed in another section of this review.

The alleviation of malignant hypertension has been the purpose of a large amount

of research based on or interpreted in terms of the assumption that phenolic pressor amines are alterably related to the genesis of this disease. Shroeder injected tyrosinase (phenol oxidase) into hypertensive rats and demonstrated a reduction in their blood pressure (36). Later he was able to extend this finding to some degree (37). His interpretation of these results was essentially that the damaged kidney was not capable of oxidizing the phenolic nucleus of the compounds and that the added tyrosinase replaced the naturally occurring but now missing phenol oxidase. This ignored the feeling of others that it is doubtful whether a true phenol oxidase capable of oxidizing tyramine or dihydroxyphenylethylamine exists in mammalian tissues (38). Prinzmetal, Alles, et al. came to the conclusion that the effect was nonspecific. Their boiled preparation of tyrosinase was as effective in lowering the blood pressure of hypertensive patients as was their active material (39). Shroeder also reported that the administration of crude amine oxidase preparations reduced the blood pressure of both hypertensive and normal rats (40), but these results are open to a similar criticism.

Attempts have been made to associate the angiotonin or hypertensin theory of essential hypertension with the phenolic pressor amine hypothesis but these have not been successful. While Croxatto and Croxatto did not make this distinction clear at first (41), they later showed that renal hypertensinase and amine oxidase were distinctly different enzymes as judged by enzyme characteristics (42). Bing, Zucker and Perkins also found angiotonin and amine oxidase to be different enzymes (43).

Still more recent is the interest in quinones as antipressor agents (44). There is no doubt that a large number of these agents, among them being 2-methyl-1,4-naphthoquinone which possesses vitamin K activity, reduce the mean blood pressure of acutely hypertensive rats without materially affecting the blood pressure of normal animals. The rationale back of this effect is stated in part by most of these reports but is discussed in papers by Oster and his associates (45). It can be shown that if a solution of an amino acid or β-phenylisopropylamine is aerated with p-cresol and phenol oxidase or ascorbic acid, deamination of the compounds occurs (15, 46). Also, the first step in the oxidation of an ortho dihydric phenylethylamine by catechol oxidase is the corresponding ortho quinone (47). Such a quinone is very unstable, being further oxidized irreversibly to melanin precursors or reduced to the corresponding catechol derivative. Thus, if the ortho quinone and β-phenylethylamine were allowed to react, the quinone would be reduced and the β-phenylethylamine finally deaminated, giving rise to the corresponding aldehyde which could tie up another atom of β-phenylethylamine in a Shiff's base arrangement, as had already been suggested (48).

So far as we know, any substance which will tend to stabilize the catechol-quinone oxidation-reduction system, so as to prevent further oxidation, will inhibit the action of a phenol oxidase on the catechol derivative and so prevent the formation of the necessary quinone. It has been reported that up to 1 gram of ascorbic acid per day for four months administered to hypertensive dogs did not reduce their blood pressure (49). This is interesting for it has been dem-

onstrated that not only does vitamin C deaminate β-phenylisopropylamine *in vitro* but the amount of the amine administered to dogs which was excreted in their urine was inversely related to the amount of ascorbic acid that was injected into the animals (46). Such experiments are not easily reconciled with the above theory.

Unfortunately the quinones are ineffective insofar as they have been tried on human cases of hypertension (50) and on renal hypertensive dogs (51). In a personal communication from Doctors Goldring, Chasis and Smith they report that 2-methyl-1,4-naphthoquinone and 1,4-cyclohexandione in dosages up to 50 mgm./kgm. were ineffective in the treatment of human essential hypertension (50). These compounds were reported by others as effective antipressor agents in rats. Doctor Goldblatt has failed to observe a hypotensive effect in dogs with experimental renal hypertension which were treated with quinones, submitted to him for the test by some investigators who had observed a hypotensive effect in rats (51). Since the quinones are not nearly so effective anti-pressor agents in those animals which are known to have amine oxidase normally present in their kidneys, it is difficult to relate their antipressor effect to the supplementation of restricted deamination in hypertensive rats having perinephric scarring but which never had the enzyme in their kidneys.

THE MODE OF ACTION OF SYMPATHOMIMETIC AMINES. Much more is known about the manner in which the body affects these compounds than about the manner in which they affect the body. There are probably as many reasons for this as there are approaches to the problem, and both have been and still are influenced by the state of knowledge of physiology or biochemistry of cellular metabolism. To describe the action of epinephrine as being on or at the myoneural junction or on the neuro-effector mechanism carries a strong morphologic connotation. However, a recent publication by Marrazzi correlating the structure of sympathomimetic amines with their action on sympathetic ganglia as measured by alterations in postganglionic potentials is an attractive approach to this phase of the problem (52). Use of the terms neurotrophic and musculotrophic, or distinguishing between sympathomimetic and pseudosympathomimetic amines on the basis of the effect of these compounds as complicated by premedication of animals with agents (cocaine, ergotoxin, etc.), whose modes of action are no better understood, serves a very limited purpose.

The efficacy with which epinephrine and its analogs acts is dependent on the functional integrity of the organs or systems directly and indirectly involved (53). The accelerating effect of epinephrine on glycogenolysis which is accompanied by an increase in cellular metabolism is a characteristic feature of its action. This glycogenolytic effect is accompanied by hyperglycemia and an increase in blood lactate (54) but the amine does not seem to influence directly the utilization of an elevated blood sugar level (55).

This hyperglycemic characteristic of epinephrine transcends in sensitivity all generally recognized sympathomimetic effects of the agent (56), and is not approximated by its most closely related pressor amines. The hyperglycemic effect of epinephrine is very definitely influenced by the adrenal cortical hormones

(53b, d) which synergize and by insulin which inhibits its effect (57). As the injection of sympathomimetic amines is increased from an infinitely small amount, the hyperglycemic response to epinephrine precedes its pressor effect (56) whereas the pressor action precedes whatever hyperglycemic effect the other sympathomimetic amines make manifest (58). These facts tempt one to wonder whether the latter agents produce an elevation in blood glucose except as they increase blood flow through the adrenal gland or otherwise indirectly influence the liberation of epinephrine.

The theory of Gaddum and Kwiatkowski (59) for the mode of action of ephedrine introduced a unique concept which was extended by others (60) to cover the action of related compounds. This hypothesis is based on the observation of Blaschko, Richter and Schlossman that ephedrine which is refractory to deamination by amine oxidase is able to inhibit the action of that enzyme on epinephrine by substrate competition (61). The formulation of the concept borrows precedent from the established competition between acetylcholine and eserine for choline esterase as the mode of action of eserine in the body (62). It was proposed, then, that ephedrine acted by inhibiting the normal inactivation by amine oxidase of epinephrine elaborated at nerve endings. Thus the effect of epinephrine was increased and prolonged. Since all sympathomimetic amines having a substituted isopropylamine side chain behave like ephedrine with respect to amine oxidase their pharmacological actions might be interpreted similarly. The hypothesis serves to explain the potentiation of ephedrine to subsequent action of adrenergic nerves (59) and the increased pressor effect when epinephrine is injected following ordinary pressor doses of any of these isopropylamine derivatives (63). The diminishing pressor effect of successive ephedrine doses, and the ability of large doses of ephedrine and related compounds to diminish the response to epinephrine, were suggested as being due to blockade of the motor receptors by ephedrine (64). Thus large doses of ephedrine could inhibit the action of epinephrine while blocking its deamination by amine oxidase.

The simplicity of this hypothesis at once makes it appealing. However the theory is probably no more secure than the suggestion that amine oxidase is responsible for the inactivation of epinephrine in the body, which does not seem to be the case, and it does not account for the very great diversity of pharmacologic effects of these agents.

The theory that the central stimulating action of amphetamine is due to the competitive inhibition of tyramine oxidation by amine oxidase thus diminishing the formation of aldehydes which depress cerebral oxidations (65) is inconclusive. Many similar amines which do not possess this property of central stimulation likewise depress the *in vitro* oxygen uptake of brain tissue. There appears to be no consistent difference between the depression of brain respiration *in vitro* by the optical forms of amphetamine used as a substrate nor is substrate competition materially influenced thereby, although there is a great difference in the central stimulating effect of the *d* and *l* forms of the compound (B).

Neither of these theories is concerned with the mode of action of epinephrine nor does either have as a major part of its thesis the possibility that the com-

pounds *per se* may be responsible predominantly for the effects they produce. Certain recent developments permit the consideration of the "receptor mechanism" or "receptor substance" as an integral part of the mechanism for contraction, secretion, etc., or as a component of the metabolic cycle responsible for such action.

For a number of reasons we have looked to an effect of the sympathomimetic amines on the carbohydrate and on the associated phosphorylation cycles as likely sites of their cellular action. The physiologic response to epinephrine and the related compounds is a dynamic one affecting the principal function of a tissue, contraction in the case of muscle.

During the past few years a great deal of research has related myosin to adenosinetriphosphatase (ATPase), and has associated the breakdown of adenosintriphosphate (ATP) with the alteration of bifringence of myosin and with muscular activity (66). Another function of the dephosphorylation of ATP is to furnish energy and phosphate for the phosphorylation of glucose (67), which process is necessary before the latter compound can undergo normal degredative procedures.

Several points of attack suggest themselves for a study of the effect of sympathomimetic amines on this trigger mechanism. Some of them have been subjected to investigation. 1. Preliminary experiments in our laboratory did not show the breakdown of ATP to ADP (adenosindiphosphate) in the presence of ATPase to be affected materially *in vitro* by these compounds. This is not surprising, for under ordinary experimental conditions it would be difficult to note other than inhibitory effects. 2. The rate or extent of phosphorylation of ADP might be effected. Govier, Bergmann and Beyer found the phosphorylation of thiamin to be increased by certain sympathomimetic amines (68). 3. The breakdown of the principal phosphate donors for the synthesis of ATP, i.e., 1,3 diphosphoglyceric acid and enol-phosphopyruvic acid, might be influenced by these compounds. 4. The oxidation of dicarboxylic metabolites which serve as a substantial source of energy for the phosphorylation of compounds (69) might be influenced by these amines. Omitting for the moment the rôle of creatinine, a marked effect of the sympathomimetic agents at any one of these points might be thought to have a similar end effect on the ATP-ATPase mechanism. Other points of attack have been omitted at present because of lack of evidence bearing on them.

The phosphorylation of thiamin in the presence of the succinate system has served as a model for determining the effect of sympathomimetic amines on the utilization of energy from succinate oxidation for phosphorylation processes. It has been demonstrated that epinephrine and certain other sympathomimetic amines are able to accelerate the oxidation of succinate and the utilization of that energy for the phosphorylation of thiamin (68b). The effect on succinate oxidation could not be demonstrated when the substrate was being oxidized in a complete and uninhibited set up, but when α-tocopherol phosphate was added to retard succinate oxidation the acceleration effect of these amines was striking. Very likely the tocopherol phosphate served to reduce the rate of succinate oxidation from its maximum to that more resembling the normal

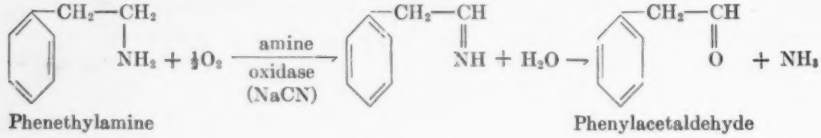
state *in vivo*. Still more recently these investigators have found that certain of these amines accelerate the α-tocopherol phosphate-inhibited oxidation of lactate (70). Bearing on this point, Houchin and Mattill found that the addition of α-tocopherol phosphate made possible the reduction of the high succinoxidase activity of vitamin E deficient muscle to normal values (71). The prevalence of this state of partial inhibition is manifested in the tonus of muscle. Much less energy would be required to alter in either direction this balanced system than need be expended otherwise.

From the above lead it would be feasible to extend such research to cover the effect of the amines on the formation of ATP as influenced by the rate of succinate and other metabolite breakdown and the efficiency with which the energy is used. This last point is a most important one, for Potter has recently stressed the fact that a high rate of oxygen uptake from succinate oxidation is no guarantee that phosphate-bond energy is thereby provided (72). Such evidence might assist materially to explain the mode of action of sympathomimetic amines. Such a concept would allow much more flexibility than would be permissible if the principal actions of the amines were mediated through a common agent, epinephrine.

THE INACTIVATION OF SYMPATHOMIMETIC AMINES. The inactivation of sympathomimetic amines seems to be much better understood than is their mode of action. Reviews bearing on this subject are those of De Meio (73), Beyer and Morrison (4e) and Hartung (74).

Amine oxidase is probably the enzyme most widely implicated in the inactivation of these compounds. It was first described by Hare (75) and was partially purified by Kohn (76). The enzyme is widely distributed in mammalian tissue (30) from which it has not been isolated completely, is cyanide insensitive but can be inhibited by urea and phenylhydrazine *in vitro* (77). It is probably the same as tyramine oxidase and adrenalin oxidase (78) and may be the same as aliphatic amine oxidase (79). It has been proposed that the name monamine oxidase be used to distinguish it from diamine oxidase (80). Some of the discrepancies in the literature relating structure to rate of deamination of these compounds undoubtedly stem from the manner of preparing the enzyme for the *in vitro* experiments. These differences are so great in many instances, notably in the case of β-phenylethylamine, as to warrant further investigation in an attempt to isolate and characterize the enzyme or enzymes.

Amine oxidase brings about the deamination *in vitro* of a large number of derivatives of β-phenylethylamines, including many having a mono or dihydric nucleus (78, 4e, 81). In the course of deamination the compound is converted into the corresponding aldehyde which may be oxidized further to the analogous phenylacetic acid unless this later step is blocked, as by cyanide. The course of this reaction is illustrated by the following equation.



Phenethylamine Phenylacetaldehyde

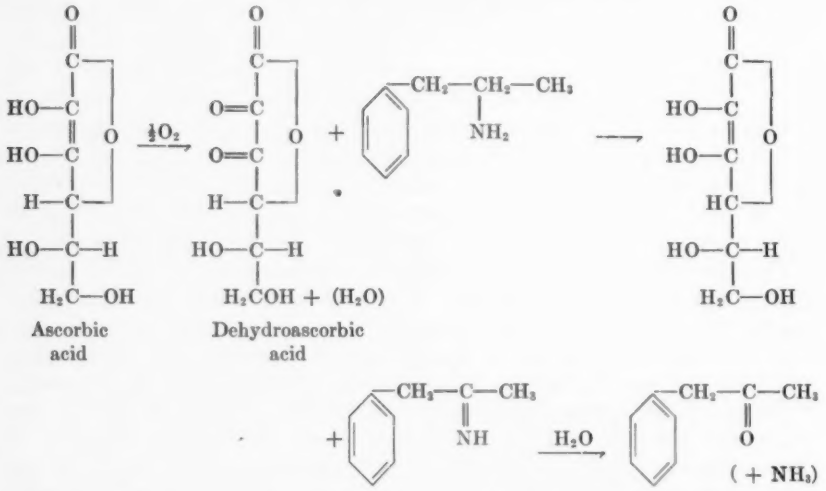
Several alterations which may be made in the side chain render the molecule partially or totally refractory to deamination. These include: (1) Positioning the amino group on any other than the terminal carbon atom (78, 81a). (2) The substitution of a single methyl group on the β-carbon atom in β-phenylethylamine does not abolish deamination, but increasing the length of that alkyl substitution or adding a second methyl group to make it a $\beta\beta$-dimethyl alteration does render the compound refractory to deamination (4e). (3) Likewise, the substitution of a single methyl group on the nitrogen atom is without qualitative effect whereas increasing the length of the substitution or the presence of two methyl groups on the nitrogen atom destroys or greatly retards the ability to be deaminated by amine oxidase (78, 81b, 4e). If the position of a single hydroxyl group on the nucleus of β-phenylethylamine be shifted from the para to the ortho position, the rate of deamination is markedly reduced (81b). The substitution of any of a number of polar groups other than hydroxyl or amino in the para position of the compound diminishes or completely inhibits deamination (B).

The presence of amine oxidase in the body, and particularly in the liver, determines whether pharmacologically active derivatives of β-phenylethylamine are effective and are excreted as such when administered by mouth. Those derivatives wherein the amino group is on the terminal carbon atom of the side chain are deaminated in the body and are neither active nor excreted as such unless a hepatotoxic agent has been administered previously (31). Those compounds which have the amino group removed from the terminal position on the side chain are not deaminated to any great extent and are active when taken orally. For the most part, they are excreted as such by man and the dog (7, 31) and to a lesser extent by rabbits (83) and rats (84).

Within rather narrow limits of structural variability there seems to be some relationship between pressor activity and rate of deamination, among those compounds which are deaminated, and also a relation between duration of action and whether or not the compounds are deaminated. More probably these are two distinct circumstances which appear to parallel over a short range, than to be causally associated. If one transgresses these very narrow limits such a relationship becomes difficult to predict or interpret. Some compounds related to β-phenylethylamine are deaminated by the enzyme and also are active pressor agents, others are deaminated but exert no pressor properties (Marfanil, 85), some are not deaminated and have good pressor qualities of long duration, while others are neither deaminated nor active as pressor amines (B).

Except for a delineation of *in vitro* results reference to mono or dihydric derivatives of β-phenylethylamine has been omitted, for amine oxidase probably plays no great rôle in their inactivation in the body (76, 86).

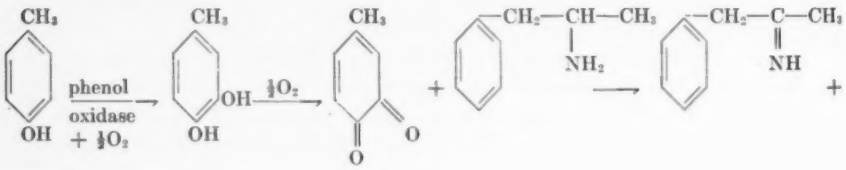
The ascorbic-dehydroascorbic acid system for the deamination of sympathomimetic amines was discovered during a search for the mechanism responsible for the inactivation of that amount of amphetamine and related compounds which was not excreted as the amine. The reaction for the deamination of amphetamine is illustrated in the following equation.

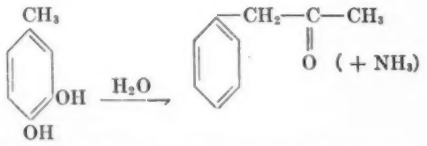


It had been demonstrated that some mechanism for bringing this about normally existed for when a hepatotoxin, carbon tetrachloride, was administered to dogs their excretion of amphetamine was increased to approximately one hundred per cent of the dose administered (82). *In vitro* it was found that primary and secondary amines were deaminated by the ascorbic-dehydroascorbic acid system whether or not the amino group was on the terminal carbon atom but the presence of a mono or diphenolic nucleus was sufficient to inhibit deamination; an oxidation-reduction equilibrium being set up between the ascorbic-dehydroascorbic acid system and the ortho dihydric nucleus, which was either initially present or formed in the course of the reactions (15). Confirmation of the functioning of this system for the deamination of amphetamine was submitted in the report that when the daily subcutaneous administration of amphetamine was held constant the recovery of the amine in the urine could be almost completely eliminated by the administration of high doses of ascorbic acid to dogs. When the ascorbic acid injections were stopped the urinary output of the amine promptly returned to the control values (46).

At least two other methods for the deamination of β-phenylethylamines having no phenolic substitutions have been reported.

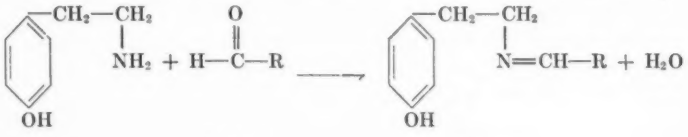
The phenol oxidase-p-cresol system has been shown to deaminate amphetamine *in vitro*. The course of the reactions as reported by Beyer is as follows (46):





The principle is similar to that reported by others. In the presence of p-cresol, phenol, or hydroquinone, phenol oxidase is capable of oxidizing glycine, phenylalanine and leucine with the liberation of ammonia (87). There is no direct evidence attesting to the physiologic significance of this system *in vivo*.

Aldehydes, as agents for the deamination of sympathomimetic amines, have been proposed by Oster and his associates (45). The principle is that aldehydes formed in the body either by deamination or otherwise are capable of forming Shiff bases with the amines and so inactivating them. Bernheim had previously suggested that such a reaction occurred in the course of deamination of amines by amine oxidase (48). The general course of the reaction is as follows:



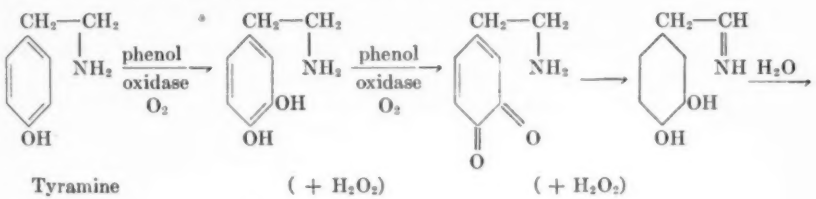
Tyramine Aldehyde Shiff base

This hypothesis lacks direct experimental support.

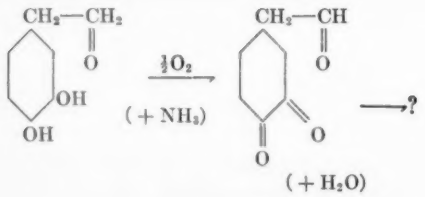
The phenol oxidases have received a tremendous amount of attention from a number of sources. They have already been mentioned under a synonym, tyrosinase, in the sections on hypertension. Most of the older ideas of epinephrine inactivation had to do with the oxidation of its catechol nucleus by such an enzyme.

There is no doubt that a number of such enzymes exist. The names phenol oxidase or cresolase and polyphenol oxidase or catechol oxidase serve to distinguish between them on a basis of substrate affinities, although the two forms almost always occur together. These enzymes can be obtained in a highly purified state. Copper is the oxidation-reduction component and the enzymes are therefore cyanide sensitive (88).

In the presence of phenol oxidase an atom of oxygen is introduced into such a molecule as tyramine adjacent to the hydroxyl group to form a catechol nucleus which in turn is oxidized to an ortho-quinone stage in the formation of melanin. The course of the reaction is believed to be as follows:



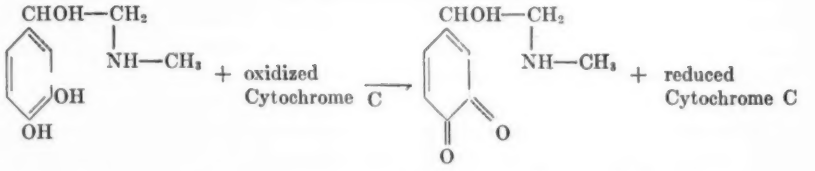
Tyramine (+ H_2O_2) (+ H_2O_2)

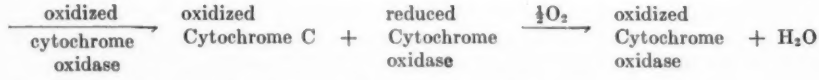


Reports dealing directly with the relation of structure to the oxidation of monohydric and dihydric phenylethylamines are those of Beyer (89), Alles (90), and Randall and Hitchings (91). The principal generalizations which can be derived are: (1) Whereas p-hydroxyphenylethylamines may be rapidly oxidized, the corresponding meta and ortho hydric compounds are refractory to oxidation on the ring. According to Randall and Hitchings (91) β-(m-hydroxyphenyl)-ethylamine is very slowly oxidized. (2) If a hydroxl group is introduced on the same carbon atom of the side chain as that bearing the p-phenolic nucleus, the rate of oxidation of the nucleus is very greatly reduced. Furthermore, if a keto group is substituted for the hydroxyl group on the β-carbon atom of the side chain, oxidation of the nucleus is abolished. (3) While somewhat the same generalizations described in (2) obtain for the compounds having a catechol nucleus the differences in rate of oxidation are not nearly so distinct.

The functional significance or even the presence of phenol oxidases in mammalian tissues is doubtful. Bloch in 1917 (92), claimed to have demonstrated the "dopa" reaction in skin in experiments wherein "dopa" was incubated with the tissue and over a period of time melanin was formed. Similarly, Pugh (93) demonstrated the "dopa" reaction in the skin of rabbits and the report was confirmed by Charles (94). Cadden and Dill have reported a polyphenolase extracted from kidney but it was more of the nature of a laccase and did not oxidize compounds of the nature with which we are concerned (95). Hageboom and Adams reported the presence of a phenolase in a mouse melanoma which oxidized tyramine (96). This is the closest to normal tissue in which such an enzyme has been shown to be present. Bhagvat and Richter have surveyed tissues from a large number of animal species and concluded that a phenol oxidase could not be demonstrated in mammalian tissues by the usual methods (38). Aside from the doubtful presence and lack of functional significance of a phenol oxidase for the inactivation of sympathomimetic amines in the mammalian body there are a number of physiological observations which support this view. These will be considered in the section pertaining to the conjugation of phenolic amines.

The cytochrome-cytochrome oxidase system which is so universally present in tissues has been shown repeatedly to oxidize epinephrine and related compounds having an ortho dihydroxy phenolic nucleus, *in vitro* (97). The equation for the reactions may be written as follows:





However, it is doubtful whether it functions in this manner in the body. Hydroquinone, catechol, and homogentistic acid which are oxidized by cytochrome C *in vitro* are excreted as such or in a conjugated form.

Conjugation appears to be the principal mode of inactivation of phenolic sympathomimetic amines. The evidence for this is strong but lacks something of the finality of the correlation of deamination by amine oxidase with inactivation or excretion of β-phenylethylamines and β-phenylisopropylamines.

The fate of monophenolic sympathomimetic amines has been particularly difficult to elucidate, owing to a lack of specific analytical methods. The first noteworthy study of the excretion of tyramine, β-(p-hydroxyphenyl)ethylamine was that of Ewins and Laidlaw who administered 500 mgm. of the material to dogs and recovered up to 25 per cent of the ingested material as p-hydroxyphenylacetic acid. Perfusion through liver and uterus oxidized tyramine to p-hydroxyphenylacetic acid, but perfusion through the heart completely destroyed the amine (98). These perfusion experiments anticipated the report of Bernheim and Bernheim that the abilities to deaminate, oxidize or conjugate tyramine, tyrosine and phenol *in vitro* were variously and independently present in different tissues. Cardiac muscle was capable of opening up the ring in these molecules (99). The administration of such large quantities of the amine as was undertaken in the above experiments (98) permits a certain reservation as to the physiologic significance of the results.

Beyer and Stutzman (100) administered 30 to 90 mgm. doses of tyramine and β(p-hydroxyphenyl)isopropylamine to both man and dog. While they did not notice any definite signs of oral activity they concluded that the inactivation of these compounds must be of such a nature as not to violate inalterably the integrity of the molecule, as would be the case for an oxidative process. This was based on the fact that following oral administration there was excreted in the urine a compound which was identical with the one ingested, as judged by pressor activity and oxidation by phenol oxidase and amine oxidase. These results might be quite reconcilable with those of Ewins and Laidlaw if measures for quantitation of results in the two instances were more satisfactory.

Just what the nature of this conjugation is remains to be established. Conjugation at either the amino or the phenolic group renders tyramine inactive as a pressor agent (B). Barger and Dale found acetoxyphenylethylamine inactive on intravenous injection (101). Loeper reported the synthesis of the sulfuric

acid ester of tyramine (102) and found it to possess no pressor activity in the cat unless first hydrolyzed to yield tyramine (103). To my knowledge their synthesis of the ester has not been repeated successfully.

Arnolt and De Meio have reported that the conjugation of phenol in liver and intestinal slices is an enzymatic process. They concluded that the process, which in itself does not involve the consumption of oxygen, requires for its activation the energy produced by reactions coupled with oxygen consumption (104). Williams has studied the relation of *o*, *m* and *p* substitutions to the conjugation of phenols (105).

Conjugation of sympathomimetic amines having a catechol nucleus is more clearly defined. Richter (106) and Richter and McIntosh (107) reported that an enzyme, sulfosynthase, was responsible for the conjugation of sulfuric acid with the catechol nucleus. Apparently the over-all reaction is as follows, although there is no evidence that conjugation necessarily is in the para position or that only one phenolic group is involved.

It was clear that when epinephrine, β(3,4-dihydroxyphenyl)ethylamine and β(3,4-dihydroxyphenyl)-β-hydroxyisopropylamine were administered orally these investigators were able to demonstrate in urine only a conjugated form of the compound which after hydrolysis yielded the original agent as analyzed chemically or biologically. Their work has been confirmed and extended recently by Beyer and Shapiro (108). Both groups of investigators are agreed that the majority of the drug administered thus is excreted in a conjugated form. Qualitatively this is independent of the mode of administration. The last is an important point and one which will need further study before this can be considered unequivocally the dominant mode of inactivation of these agents. The reason for this is that the intestine and liver are the principal sites of phenol conjugation of epinephrine.

Several investigators have studied recently the conjugation of phenols with sulfuric acid (109).

Before ceasing the discussion of conjugation as a mode of inactivation it might be well to point out that on the whole conjugation of the amino group has been neglected, possibly prematurely. Snyder, Goetze and Oberst have just reported that the rat is capable of conjugating ephedrine and β-hydroxy-β-methyl-β-phenylethylamine. The total recovery of ephedrine was increased by as much as 40 per cent and the latter compound by as much as 80 per cent when the urine of the animals was hydrolyzed (84). Although this may be a species characteristic, the observation is an interesting one.

Renal excretion of sympathomimetic amines is dependent on their mode of

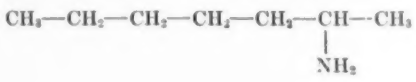
inactivation in the body. Thus this section can be made to serve as a summary of the salient points in the preceding paragraphs.

If the nucleus is unsubstituted by hydroxyl groups, derivatives of phenylethylamine which have the amino group on the terminal carbon atom are deaminated in the body and are not excreted as such. Where the amino group is not on the terminal carbon atom the compound is refractory to deamination, except by systems ordinarily of secondary importance, and are excreted for the most part as such. Para-hydroxy derivatives of β-phenylethylamine, regardless of the position of the amino group on the side chain, are inactivated in the body but are conjugated at least in part in so loose a manner as to be indistinguishable from the free form of the compounds after excretion. Compounds having a catechol nucleus are excreted predominantly in a conjugated form when administered orally. This is qualitatively independent of the mode of administration. On hydrolysis of the urine the free form of the sympathomimetic amine is hydrolyzed from its conjugate.

THE RELATION OF STRUCTURE TO PHARMACOLOGIC ACTION. The purpose of this section is to indicate in general the quantitative and qualitative changes in pharmacologic properties that have been found to accompany basic and systematic alterations in the structure of sympathomimetic agents. In some instances these compounds are so divergent in structure as to have in common only certain pharmacologic properties; in other instances the alterations in function induced by the compounds are so at variance that their chemical structures are the greatest point of similarity.

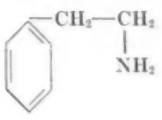
The following formulae illustrate several of the type structures and alterations which will be discussed in this section. The list is not complete but may serve as a guide to those not familiar with the nomenclature of such compounds.

Aliphatic amines:

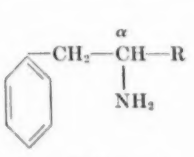


2-Aminoheptane "Tuamine"

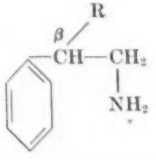
Phenethylamine:



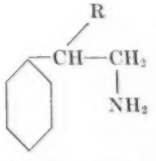
Modifications of the phenethylamine side chain:



$R = C_nH_{2n+1}$ etc.
α – substitution



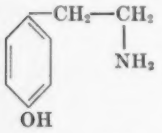
$R = C_nH_{2n+1}$ etc.
β – substitution



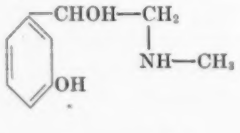
R = OH or O
aliphatic hydroxyl or keto substitution

Substitutions into the phenyl nucleus:

a. Monohydroxyphenylethylamines

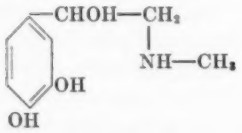


"Tyramine"
para-hydroxyl
radicle



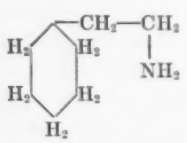
"Neosynephrin"
meta-hydroxyl
radicle

b. Dihydroxyphenylethylamines

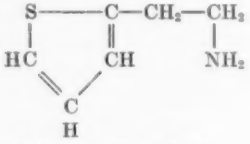


"Epinephrine"
3-4 dihydroxy
substitution

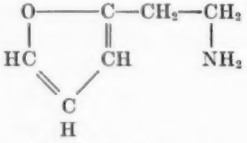
Other types of aromatic nuclei:



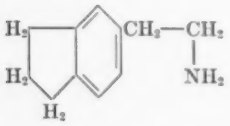
β-cyclohexylethylamine



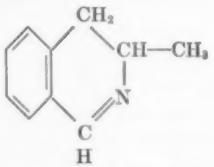
β-(2-thienyl)ethylamine



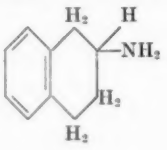
β-(2-furyl)ethylamine



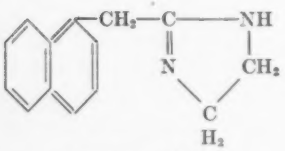
β-(5-hydrindenyl)
ethylamine



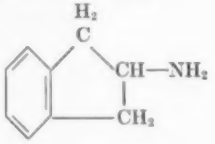
3-methyl-3,4 di-
hydroisoquinoline



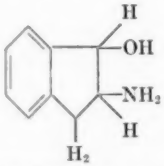
β-tetrahydro-
naphthylamine



2-naphthyl-(1')-
methyl imidazoline
"Privine"



2-aminoindane



1-hydroxy-2-amino-
hydrindene

Aliphatic amines. The recent reports on aliphatic amines, as vasoconstrictor (110) and as antispasmodic agents (111), have served to renew interest in this group of compounds. Actually the first critical investigation of a series of 21 alkylamines was that of Barger and Dale (101). Their series included the normal monoalkylamines, a number of iso-monoalkylamines from methylamine to undecylamine, and representative di, tri and quartenary ammonium compounds. The lowest members of the group were found to be depressor agents or to have no effect on blood pressure (101, 112). Definite pressor activity was attributed to n-butylamine; maximal pressor activity of the compounds was resident in

n-hexylamine. For compounds of greater chain length pressor activity decreased progressively although toxicity increased.

Rohrmann and Shonle have synthesized and studied pharmacologically (113) the most systematic group of aliphatic pressor amines to date. They confirmed the observation of Barger and Dale that, of the n-l-alkylamines, l-aminohexane had the greatest pressor effect. Positioning of the amino group on the second carbon atom of heptane or octane gave better pressor results than substitution on any other carbon atom. 2-Amino-4-methyl hexane, reportedly the most active compound of their series, and 2-amino-4-methyl heptane were more active than either of their 3-methyl or 5-methyl counterparts.

As a pressor agent the best of the aliphatic amines is less active than ephedrine (113b) and they all demonstrate tachyphylaxis (101, 110c, 114).

The phenylethylamine structure. Recalling that 1-aminohexane has a fair degree of pressor activity it is interesting that cyclohexylamine possesses only about $\frac{1}{3}$ its pressor potentialities. Cyclohexylmethylamine is only slightly more active than cyclohexylamine, but when the amino group is further removed from the ring by one carbon atom the resulting β-cyclohexylethylamine has an activity sixfold greater than cyclohexylmethylamine and almost as great as for ephedrine. β-cyclopentylethylamine has practically the same pressor activity as β-cyclohexylethylamine (113b). There is another interesting point in these data of Rohrmann and Shonle that bears reiteration at this time. 2-Amino-4-methylhexane and 2-amino-4-methylheptane were about four times as active as 2-amino-3-methylhexane and 2-amino-3-methyl-heptane, although all the compounds were less active than β-cyclopentylethylamine or β-cyclohexylethylamine. In both types of compound the amino group is one carbon atom removed from that bearing the ring, or from the actual branching of a long chain.

Barger and Dale (101) came to the conclusion that the aromatic nucleus should be one carbon atom removed from the amino group on the side chain. Whereas aniline had no pressor effect, benzylamine was slightly active. α-Phenylethylamine was still more active while β-phenylethylamine had maximal activity and γ-phenylpropylamine was again less active. Although there have been many substantiations of this fundamental point one of the best was that of Hartung and Munch (115) who studied the pressor effects of four isomeric phenylpropylamines. Actually this beta relationship of the amino group to the aromatic nucleus transcends this group of compounds and appears in other series as well.

Modification of the aliphatic side chain. If one substitutes an alkyl chain, exceeding a methyl group, or an aryl group on either the alpha or beta carbon atom the sympathomimetic effect of the resulting agent is markedly reduced, abolished, or reversed. This has been found to be true for substitutions in the beta position with respect to the amine by Warren et al (116), and Beyer (B) using compounds of the phenylisopropyl series synthesized by Suter and Weston (117).

Schaumann found that if the side chain of epinephrine was increased to four carbon atoms, the compound was no longer pressor but was depressor in char-

acter (118). Whereas β-phenyl-β-hydroxyisopropylamine is quite active as a pressor agent the corresponding phenylbutanolamine has little effect (119). Extending this series results in compounds which increase instead of decrease tonus of the intestine (B).

The substitution of a single methyl group on the β-carbon atom results in a marked decrease in pressor ratio, as can be demonstrated by comparing β-phenylisopropylamine with β-phenyl-β-methylisopropylamine (116) and β-phenylethylamine with β-phenyl-β-methylethylamine (B). Substitution of a single methyl group on the α-carbon atom of β-phenylethylamine confers on that compound special characteristics of long duration of action, oral activity, strong central nervous system stimulating effects without as great an alteration of pressor ratio as when substituted in the beta position. These characteristics of long duration of action and oral efficacy were reported for closely related analogs of α-methyl-β-phenylethylamine at about the same time by Chen, Wu and Hendriksen (120) and Piness, Miller and Alles (121). An interpretation of this general effect has been presented in a previous section of this review. Gunn reported that phenylallylamine had a pressor potency equivalent to β-phenylethylamine (122).

It is surprising that so few satisfactory data are available pertaining to the effect of hydroxyl or keto substitution on the β-carbon atom of the side chain on the pharmacological properties of β-phenylethylamines and β-phenylisopropylamines. For the comparisons to be strictly valid it should not be made for compounds having hydroxyl-substitutions in the aromatic nucleus. The reason for this is that the keto group markedly, and the alcoholic group to a lesser extent, influence the reactivity of the phenolic nucleus (89). The addition of the aliphatic hydroxyl group definitely decreases the toxicity of β-phenylethylamine (123) and β-phenylisopropylamine (120) and decreases their central stimulating effect (123). The compounds bearing the hydroxyl group appear to be the more active pressor agents (120, 124). This is probably due to their stronger vasoconstrictor action, as Morton and Tainter demonstrated (63c). There is no doubt that arterenol is much more active as a pressor agent than is β-(3,4-dihydroxyphenyl)ethylamine (125) or that epinephrine is more active than is β-(3,4-dihydroxyphenyl) ethylmethylamine (124b) but their toxicities are also greater.

From what little is known about the aminoketones it may be concluded that they are pharmacodynamically less active than their counterparts with or without an aliphatic hydroxyl group as judged by pressor effect or toxicity (101, 120).

Substitution of an alkyl group on the amino nitrogen of the side chain almost invariably reduces the activity of the resulting compound, regardless of the length or symmetry of the substituent. Although there are a few exceptions to this, the generalization has been repeatedly affirmed since the initial report of Barger and Dale (101). This is true for derivatives of β-phenylethylamine (B), β-phenylisopropylamine and ephedrine (127), epinephrine (128) and others (4f).

Noteworthy compounds among these are: N-methyl-β-phenylisopropylamine

(desoxyephedrine, pervitin) which stimulates the central nervous system to about the same or a greater extent than does β-phenylisopropylamine, depending on the author (127c, 129); cinnamylephedrine is reported to have local anesthetic properties (130), and certain others are claimed to have some analgesic effect (131); the N-ethyl derivatives of ephedrine (127b) and epinephrine (132) are reported to have good bronchodilator effect although their pressor activities are considerably lower than those of the parent compounds.

Alteration of the aromatic nucleus. If a single hydroxyl group is introduced into the ring, the o-hydroxy compound is usually the least active, the m-hydroxy the most active and the p-hydroxy derivative is intermediate but nearer the m-hydroxy in its potentialities. The general order of pressor activity of these monohydric amines is on the whole much greater than for the nonphenolic compounds, ranging roughly from $\frac{1}{10}$ to $\frac{1}{100}$ as active as epinephrine for the phenolic and $\frac{1}{100}$ to inactivity for the nonphenolic. The observation of the relationship of position of the hydroxyl group to activity again goes back to the report by Barger and Dale (101). In the most recent report on the subject the relative inactivity of the ortho and the superior effectiveness of the meta hydroxy compounds are borne out (133). Although Barger and Dale did not appreciate the difference in activity of the meta and para compounds, a sufficiently large number of reports testify to the unequivocally greater activity of the meta hydroxy compounds (127d, 118, 134). In general the meta and para hydroxy compounds are more toxic than are their nonphenolic counterparts, they have a greater effect on intestinal activity, they are not as active by oral administration, they have a somewhat greater bronchodilator action, and they do not exhibit tachyphylaxis. In all these respects the meta hydroxy compounds are superior. Moreover the latter group more nearly simulates the actions of epinephrine and its related agents having a catechol nucleus. Its pressor action is reversed by ergotamine and potentiated by cocaine (126), pancreatic secretion is stimulated (135), and, in the case of β(3-hydroxyphenyl)-β-hydroxyethylmethylamine, it is the only type of highly active pressor agent which does not exaggerate the cardiac irregularities potentiated by cyclopropane anesthesia (136).

If two hydroxyl groups are introduced into the nucleus, the pressor activity of the compound depends on their relation to each other and to the side chain. Dakin was impressed sufficiently by the appearance of the catechol nucleus in epinephrine to consider it to be the essential part of the epinephrine molecule (137). There is no doubt that for maximal effectiveness, regardless of the nature of the side chain, the ortho dihydroxy substitution must be in the meta and para positions. These are the so-called true sympathomimetic amines from a pharmacologic standpoint. Their pressor ratios run roughly from equal, or better, to $\frac{1}{50}$ of that of epinephrine. None of them is active as a pressor or bronchodilator agent in moderate dosage except by parenteral administration, their duration of action is relatively brief, and they are quite unstable agents. Some idea of the essentiality of the 3,4-dihydroxy nucleus may be gained by a comparison of epinephrine pressor ratios of a number of β(di-hydroxyphenyl) propylamines re-

ported by Graham, Cartland and Woodruff (4f): 2,3 dihydroxy = 1/750; 2,4 dihydroxy 1/1100; 2,5 dihydroxy 1/4000; 2,6 dihydroxy = depressor; 3,4 dihydroxy = 1/40 and 3,5 dihydroxy = 1/120.

Of all the sympathomimetic amines, arterenol, β(3,4-dihydroxyphenyl)-β-hydroxyethylamine is undoubtedly more active than epinephrine. It has been proposed to be the hypothetical "sympathin" (21).

The methoxy-phenylethylamines are among the most frequently studied of the compounds having substitutions in the aromatic nucleus. The mono, di and tri methoxy and methylene-dioxy derivatives of the more common β-phenylethylamine and β-phenylisopropylamine structures have been made. In general these compounds are more toxic and less active as pressor agents than are the corresponding phenolic or catecholic derivatives. The derivatives of β-phenylisopropylamine and especially β-(3,4-methylenedioxyphenyl) isopropylamine initiate symptoms of central nervous system stimulation when introduced into rats, but none of these agents has gained acceptance. The most comprehensive pharmacological studies of these compounds were undertaken by Gunn and his associates (138).

Two co-operative projects of considerable magnitude in this field are the pharmacologic actions described by Hjort (139) for 22 derivatives of N-methyl-β-phenylethylamine out of a group prepared by Buck (140); and the pharmacologic actions of the methoxy phenylpropylamine series described by Graham and Cartland (4f, 133), the compounds being synthesized by Woodruff and his associates (141). Graham and Cartland have reported that in some instances the methoxy compounds showed superior bronchodilator activity with less pressor effect than the corresponding hydroxy compounds. Mescaline, β-(3,4,5-trimethoxyphenyl)-ethylamine, has most interesting central nervous system stimulating properties but no notable pressor action (142).

One other type of substitution into the nucleus deserves mentioning. The β-aminophenylethylamines are an interesting group, although little is known of them. They more nearly simulate the pressor activity and lack of toxicity of the phenolic type compounds than any other substitution into the nucleus. Ephetonal, p-amino ephedrine, is less than half as toxic as ephedrine, and is a good pressor compound (132b, 143). Its effects on pancreatic secretion (135) and as a bronchodilator agent (132b) have been described. A number of similar compounds have been reported in the literature (144) and on the whole they conform to the above generalization.

Although a number of other groups have been introduced into the benzene ring, none of them yields compounds of any great interest. These include the halogens (145), esters and alkyl groups (125, 146), nitro group (125), and a number of others. Probably a fair index of the relative effect of a number of these substitutions into the nucleus on the pressor activity of a basic compound are the following epinephrine pressor ratios obtained under strictly comparable circumstances for substituted phenyl derivatives of β-phenyl-β-hydroxyisopropylamine (B): m-hydroxy 1:25, p-amino 1:45, o-fluoro 1:80, m-fluoro 1:102, p-hydroxy 1:103, m-chloro 1:133, the parent compound 1:185, p-fluoro 1:275, p-methyl 1:353, p-chloro 1:550, p-methoxy 1:800.

The substitution of other aromatic nuclei for the benzene ring leads to compounds whose actions vary from pressor to no effect to strong depressor action. A few examples will serve to illustrate this diversity. The simplest alteration is from the benzene to the cyclohexyl ring. Recent reports along this line are those of Rohrmann and Shonle (113b) and Lands, Lewes and Nash (147).

The cyclohexyl and cyclopentylethylamines and isopropylamines have slightly less pressor effect, are volatile, but do not have the pronounced central nervous system stimulating effect of their phenyl homologs. The isosteres of the phenylethylamines are interesting compounds. The closest are the β-thienylethylamines. These have been studied by Tainter (140b), Alles and Feigen (148), Warren, Marsh, Thompson, Shelton and Becker (116), and Van Zoeren (149). Of all the heterocylic derivatives of the ethylamines these more nearly resemble the β-phenylethylamines pharmacologically. Replacement of the sulfur by oxygen in the heterocylic ring yields the β-furylethylamines (148) which are less active than the β-thienylethylamines. One other heterocylic compound which has the same pressor ratio as β-phenylethylamine is β-(5-hydrindenyl) ethylamine (B).

Other types of nuclei which have not yielded compounds of noteworthy sympathomimetic effects are, α,β-diphenylethylamines (150), isoquinolines (151), 1-hydroxy-2-aminohydrindene (152, 153), β-tetrahydronaphthalylamine (101), and indanamines (153). The most successful of all researches in this direction are those reported by Scholz (4b) and recently by others (154) for certain imidazolines of which "Privine" is the best known example. Histamine is an example of a heterocylic substituted β-ethylamine which is an intensely depressor agent. On the other hand an analog of tryptamine, the quarternary trimethylindole-ethylamine iodide (155), like so many quarternary ammonium compounds (101, 156), is a pressor agent.

Many more compounds more or less resembling the structure of sympathomimetic amines have been prepared and studied. On the whole they have served no outstandingly useful purpose in this field and so will not be reviewed here. Reference to them can be found in the chemical literature.

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[1] In a number of instances the author has referred to work done in this laboratory which for lack of time has not yet been prepared for publication.

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THE FIXATION OF CARBON DIOXIDE AND THE INTERRELATIONSHIPS OF THE TRICARBOXYLIC ACID CYCLE

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It is proposed in this communication to consider the mechanism of carbon dioxide utilization by animal tissue together with those problems which are closely related to this subject. The fixation of carbon dioxide occurs in the synthesis of glycogen and is intimately involved in biological oxidations through the tricarboxylic acid or Krebs cycle. This cycle constitutes a mechanism by which carbohydrate and fat may be oxidized. These closely interwoven problems make up the main task of this review.

The early developments in this field do not need to be considered extensively since the material has been adequately reviewed by others (1), (2), (3). In the ten years which have elapsed since Wood and Werkman (4) first demonstrated carbon dioxide fixation by heterotrophic bacteria, there has been rather rapid advance. This progress has been especially rapid since 1940, when general

agreement was reached that carbon dioxide can be used by most heterotrophic forms of life. The acceptance of this phenomenon stemmed mainly from the fact that isotopes of carbon became available, which made possible the active study of utilization of carbon dioxide by organisms other than the special bacteria with which the first observations were made. With isotopes it proved easy to demonstrate that many organisms can assimilate carbon dioxide; bacteria (5), (6), molds (7), protozoa (9), and animals (10), (11), (12), (13). After completion of this step, investigators were confronted with the more difficult task, that of solving the chemistry underlying the reactions by which the basic conversion of carbon dioxide to an organic linkage is brought about. A good start has already been made in this direction but much still remains obscure. Beyond the direct object of obtaining information concerning the chemical reaction lies the goal of interpreting the function of carbon dioxide fixation in biochemistry. At present such considerations are largely speculation.

The studies of carbon dioxide fixation have led, and will lead, not only to a better understanding of this process, but also, in an indirect way, to the solution of other problems. Already these investigations have contributed to the understanding of the mechanisms of the tricarboxylic acid cycle and of glycogen synthesis. Ultimately it is to be expected that the information will constitute a real contribution toward clarification of the chemical reactions of photosynthesis. It is quite probable that the underlying reactions may be similar in the two processes, the essential difference being that the required energy is radiant in photosynthesis and chemical in the case of heterotrophic metabolism (14), (15), (16).

SECTION I. INITIAL REACTIONS IN THE FIXATION OF CARBON DIOXIDE. Until recently there was only one example of a reaction by which carbon dioxide was believed to be fixed in an organic linkage by heterotrophic forms of life. This reaction consisted of the combination of pyruvic acid and carbon dioxide, to form oxalacetate (17). The kinetics and intermediate steps of the reaction are still obscure. Recently, in addition to this example of fixation by C_3[1] and C_1 combination, evidence has been presented of C_2 and C_1 addition (18), (16) and very recently of C_5 and C_1 combination (19). As yet C_2 and C_1 addition has not been demonstrated with animal tissues. The biological significance of the last two reactions has received practically no consideration.

There is at present no reason to believe that other reactions of carbon dioxide fixation will not be observed; there are many decarboxylations which have not yet been tested for reversibility. Certainly there is no longer any basis for assuming that C_3 and C_1 addition is the key reaction for all carbon dioxide fixation. The relative importance of the fixation reactions in biochemistry cannot be judged at present. It is conceivable that some of the fixations are not essential for formation of necessary compounds, but that they are simply the result of the reversibility of breakdown mechanisms.

There are three known examples of primary fixation reactions. By primary fixation is meant the reaction in which the carbon to carbon linkage is first

[1] Compounds containing n number of carbons will be referred to as C_n.

formed. This is in distinction to the overall changes following this initial fixation of carbon dioxide. Presumably the primary fixation reaction initiates fixation which ultimately leads to the inclusion of carbon from carbon dioxide in a large number of derived compounds.

The three primary fixation reactions will be considered in order of their discovery.

C_3 and C_1 addition by oxalacetate β-carboxylase.

(1) $$C^{13}O_2 + CH_3 \cdot CO \cdot COOH \rightleftharpoons C^{13}OOH \cdot CH_2 \cdot CO \cdot COOH$$

Reaction 1 was first proposed as a working hypothesis for the fixation of carbon dioxide in the propionic acid fermentation (20). This proposal was based on the observation that in the fermentation of glycerol by the propionic acid bacteria the succinate formed and carbon dioxide utilized were approximately equimolar. This fact pointed to a union between carbon dioxide and a C_3 compound formed from the three carbons of glycerol. Pyruvic acid had been isolated from this fermentation (21) and was proposed as the C_3 compound.

No real progress was made toward the identification of the actual components of this reaction until Krampitz and Werkman (22) discovered a heat-labile enzyme from *Micrococcus lysodeikticus* which catalyzed the decarboxylation of oxalacetate to pyruvate. Later, with $C^{13}O_2$ it was possible to show that the reaction was reversible and that the enzyme could induce the incorporation of carbon dioxide into oxalacetate (17). The C^{13} of the labeled carbon dioxide was found exclusively in the carboxyl adjacent to the methylene group of oxalacetate as predicted in Reaction 1. Since the only compounds added in this reaction were oxalacetate and carbon dioxide, and pyruvate and carbon dioxide were the only products formed, the evidence was fairly clear that the carbon dioxide was introduced into the oxalacetate with no alteration of the compounds other than perhaps minor transformations for activation such as to form phosphorylated compounds.

With animals a similar type of direct evidence of fixation of carbon dioxide by oxalacetate β-carboxylase was not obtained although claims for proof of such a reaction were made on the basis of indirect results. These claims have already been critically examined in a previous review (2).

Evans *et al.* (23) in 1943 made an important contribution toward the proof that animal tissue fixes carbon dioxide by oxalacetate β-carboxylase, when they obtained a cell-free preparation from pigeon liver containing this enzyme. The enzyme was heat-labile and catalyzed the decarboxylation of oxalacetate to pyruvate, just as the bacterial enzyme (22), but differed in one apparently minor respect in that it was activated only by Mn^{++} instead of either Mn^{++} or Mg^{++}. When both fumarate and pyruvate were substrates, the liver extract catalyzed a rapid fixation of carbon dioxide, as judged from the amount of C^{11} in organic linkage when $C^{11}O_2$ was used as a tracer. The net chemical change during this reaction consisted in a conversion of the fumarate to lactate and carbon dioxide. The original pyruvate concentration remained unchanged during the reaction but very little $C^{11}O_2$ was fixed if the pyruvate was omitted from the reaction mixture.

When pyruvate was the only added substrate and crude extracts were used there was considerable fixation of carbon dioxide, but with dialyzed or purified enzymes the C^{11} fixed was reduced to a very small value. The fixed C^{11} in the latter case was almost quantitatively precipitated as dinitrophenylhydrazone (presumably of pyruvate). Evans *et al.* (23) have considered this fixation of carbon dioxide with pyruvate to be an uncomplicated example of the primary fixation by Reaction 1. Since the amount of fixation was reduced by purification of the enzyme it is possible that necessary unknown reactants were removed by the procedures of purification. This fixation may, thus, have the same character as the fixation with mixed substrates of pyruvate and fumarate in that it is not a simple reaction. The results were, thus, not a very satisfactory demonstration of the primary reaction.

Evans *et al.* (23) proposed that the fixation with pigeon liver extract occurred by reversibility of the following reactions:

(2) $$C^{11}O_2 + CH_3 \cdot CO \cdot COOH \rightleftharpoons C^{11}OOH \cdot CH_2 \cdot CO \cdot COOH$$

(3) $$C^{11}OOH \cdot CH{:}COH \cdot COOH \rightleftharpoons C^{11}OOH \cdot COH{:}CH \cdot COOH \quad \text{(Shift of OH)}$$

(4) $$COOH \cdot CH{:}CH \cdot COOH + H_2O \rightleftharpoons COOH \cdot CH_2 \cdot CHOH \cdot COOH$$

(5) $$COOH \cdot CH_2 \cdot CHOH \cdot COOH + CH_3 \cdot CO \cdot COOH \rightleftharpoons COOH \cdot CH_2 \cdot CO \cdot COOH + CH_3 \cdot CHOH \cdot COOH$$

Reactions 2 and 3 were proposed to account for the fixation when pyruvate was the only substrate and Reactions 2 through 5 when both pyruvate and fumarate were included. Krampitz *et al.* (17) have demonstrated that the shifting of the OH of enol-oxalacetate does not occur spontaneously via Reaction 3. They showed that excess C^{13} in one carboxyl group of oxalacetate did not become spontaneously distributed in both carboxyl groups of oxalacetate and therefore the possibility of Reaction 3 occurring non-enzymatically was excluded.

In order to further verify the reactions, Wood, in collaboration with Evans and Vennesland (24), studied the fixation in greater detail. Carbon dioxide labeled with C^{13} was used and the components of the reaction were degraded for determination of the location and concentration of the C^{13} in each compound. It was demonstrated that the C^{13} was entirely in the carboxyl groups of the pyruvate, lactate, malate and fumarate and that the concentration of C^{13} was approximately the same in all the carboxyl groups. These results were, therefore, in complete agreement with the proposed Reactions 2, 4, and 5 of Evans *et al.* (23). However, an attempt was not successful to verify by direct methods that the liver oxalacetate β-carboxylase induced exchange of carbon dioxide in oxalacetate, in a manner similar to the bacterial preparation. These studies indicated that the reversal of Reaction 1 was apparently too slow to account for the rapid exchange observed with fumarate and pyruvate. There thus remained some doubt as to the mechanism of the primary reaction of the fixation by the pigeon liver extract.

This uncertainty has largely been removed by the recent experiments of Utter and Wood (25). They have demonstrated that the decarboxylation of oxalace-

tate by the liver extract is reversible, when adenosine triphosphate (ATP) is added to the enzyme. With $NaHC^{13}O_3$, oxalacetate, no ATP and the enzyme of liver extract a very slow exchange of C^{13} in the oxalacetate was observed. When ATP was added to the mixture the rate of fixation was comparable to that observed with pyruvate and fumarate. Adenylic acid did not replace ATP in this reaction, nor did cocarboxylase. Mn^{++} is a necessary activator for the carboxylation and Mg^{++} will not replace the function of the Mn^{++}, just as has been previously observed for the decarboxylation (23). Whether or not the requirement of ATP is a point of difference from the bacterial oxalacetate β-carboxylase is not apparent at present. It is possible that there is sufficient ATP in the bacterial preparation to supply the requirements of the reaction.

The function of the ATP in Reaction 1 is not evident. It remains to be investigated whether ATP serves to phosphorylate oxalacetate or pyruvate and these esters are the active components of the reaction, or whether, for example, ATP acts in the synthesis of an essential phosphorylated coenzyme as Gunsalus *et al.* (26) have observed with ATP and pyridoxal in their studies of tyrosine decarboxylase. The function of the ATP is not to phosphorylate thiamin since cocarboxylase does not catalyze the exchange. Neither is its function to phosphorylate pyruvate to give phosphopyruvate. Phosphopyruvate will not replace the ATP function.

It is interesting in this connection that Kalnitsky and Werkman (27) with an extract of *E. coli* have observed the formation of oxalacetate or a chemically similar compound from pyruvate and bicarbonate under anaerobic conditions. The oxalacetate was determined by the aniline citrate reaction and by the colorimetric method of Straub (28). However, Evans (1), on the basis of thermodynamic data, has calculated for the direct carboxylation of pyruvic acid, that the maximum amount of oxalacetate that could be formed would be only 0.06 per cent of the observed amount. He has concluded that either the pyruvate forms some derivative of higher energy content which is the true substrate of carboxylation, or the bulk of the oxalacetate found was derived from another source. Apparently Evans (1, p. 281) misinterpreted Kalnitsky and Werkman's report, however, for although the latter authors speak of direct carboxylation they were not unaware that energy is necessary for the reaction. They considered this aspect of the problem and suggested that the simultaneous formation of acetylphosphate from pyruvate by the *E. coli* extract might be the logical source of energy for the endergonic reaction. The finding by Utter and Wood that ATP is an essential component of the reaction gives some support to the contention that there may be a high energy derivative with which the actual carboxylation occurs.

C_2 and C_1 addition by the phosphoroclastic reaction. Early attempts to prove that there was fixation of carbon dioxide by C_2 and C_1 addition were not successful. Krampitz *et al.* (17) with $C^{13}O_2$ were unable to detect any reversibility in the oxidative decarboxylation of pyruvate with *Micrococcus lysodeikticus;* the C^{13} was determined in the carboxyl of the pyruvate remaining after fifty per cent of it had been oxidized by the bacteria. Evans (139) obtained similar negative

results with yeast carboxylase but Carson *et al.* (29) reported a very small positive value. Because the pyruvate was separated as the 2,4-dinitrophenylhydrazone, which is not specific for this compound, and because the amount of C^{11} fixed was so small the significance of the latter result is uncertain.

Nevertheless, there was some evidence in certain bacterial fermentations that C_2 and C_1 combination did occur. Slade *et al.* (5) found that *Clostridium welchii* and *Cl. acetobutylicum* produced lactate in which $C^{13}O_2$ was fixed in the carboxyl group. Since there was no detectable amount of C_4 dicarboxylic acids formed in these fermentations there was no evidence that the C^{13}-lactate arose *via* C_3 and C_1 fixation. Also, Brown *et al.* (30) could find no evidence that butyl alcohol bacteria dissimilated C_4 dicarboxylic acids. This finding made still more remote the possibility that the C^{13}-lactate was formed from these acids.

The above results were suggestive but were by no means conclusive. The proof of C_2 and C_1 addition came from the study of a specific enzyme reaction, the phosphoroclastic split of pyruvate, which was first demonstrated by Utter and Werkman (31) with an extract from *E. coli*.

(6) $$CH_3 \cdot CO \cdot COOH + H_3PO_4 \rightleftharpoons CH_3 \cdot CO(OPO_3H_2) + HCOOH$$

The background of this discovery was as follows:

Lipmann's important investigations of pyruvate oxidation with *Lactobacillus delbrückii* had pointed to acetyl phosphate as a key intermediate in metabolism (32), especially since the phosphate linkage in acetyl phosphate is of the energy-rich type (33) and is able to bring about the phosphorylation of adenylic acid. Curiously, the phosphoroclastic reaction was first suggested as the result of a misunderstanding. Silverman and Werkman (34) in an investigation of the conversion of pyruvate to acetyl-methylcarbinol by *Aerobacter*, presented evidence that phosphate was necessary for the reaction. Lipmann (33, p. 135) believing that they were working with the so-called hydroclastic split of pyruvate (Reaction 7)

(7) $$CH_3 \cdot CO \cdot COOH + HOH \rightarrow CH_3 \cdot COOH + HCOOH$$

and that phosphate had been found necessary for the reaction, suggested that the hydroclastic split might in reality be a phosphoroclastic split (Reaction 6).

Kalnitsky and Werkman (35) later found that cell-free extracts of *E. coli* were unable to dissimilate pyruvate after a short period of dialysis and that the system was reactivated by addition of phosphate. The principal conversion was to acetic and formic acids, although succinate and lactate were also formed. Utter and Werkman (31) then provided convincing proof of the phosphoroclastic reaction by showing that one mole of labile phosphate (assumed to be acetyl phosphate) arose for each mole of pyruvate converted to a C_2 compound by an *E. coli* extract. Most of the acetyl phosphate was formed by the phosphoroclastic split but apparently about 15 per cent was formed by dismutation of the pyruvate to lactate and acetyl phosphate and carbon dioxide.

In the phosphoroclastic cleavage of pyruvate the energy from the splitting is largely preserved in the acetyl phosphate. The external energy loss from the

system is thus sufficiently low that the reaction becomes much more reversible than would be possible with the hydroclastic split (36). Utter *et al.* (18) were able to demonstrate this reversibility by dissimilating pyruvate with the *E. coli* extract in the presence of $HC^{13}OOH$. The residual pyruvate was shown to contain excess C^{13} in the carboxyl group. For example, after sixty minutes' anaerobic incubation, thirty per cent of the pyruvate was broken down and the formate contained 0.77 per cent excess C^{13} and the carboxyl of the pyruvate 0.78 per cent. Thus, the excess C^{13} which had been added as formate was in complete equilibrium with the pyruvate-carboxyl. Since the carbon dioxide in the system at the conclusion of the experiment contained only 0.06 per cent excess C^{13}, it is clear that the heavy carbon pyruvate could not have resulted from oxalacetate β-carboxylase fixation of carbon dioxide, or any reaction involving carbon dioxide as such.

When pyruvate was dissimilated in the presence of $CH_3C^{13}OOH$ and adenosine triphosphate the residual pyruvate contained excess C^{13} in the carbonyl position. This exchange was about twenty times slower than that with pyruvate and formate, apparently because Reaction 8 was limiting.

$$(8) \qquad CH_3 \cdot C^{13}OOH + ATP \leftrightharpoons CH_3 \cdot C^{13}O(OPO_3H_2) + ADP$$

$$(9) \qquad CH_3 \cdot C^{13}O(OPO_3H_2) + HCOOH \leftrightharpoons CH_3 \cdot C^{13}O \cdot COOH + H_3PO_4$$

The reversibility of Reaction 8 has been shown by Lipmann (37).

Although the proof that fixation occurs as pictured in Reaction 6 is quite conclusive, perhaps one final piece of evidence is desirable to show that it is acetyl phosphate rather than another derivative of pyruvate which is the component of the rapid equilibrium with formate. This proof would involve determining whether or not heavy carbon acetyl phosphate equilibrates with pyruvate as rapidly as labeled formate.

Lipmann and Tuttle (16) have extended the investigations of the equilibrium of the phosphoroclastic reaction using acetyl phosphate and formate and determining chemically the pyruvate in equilibrium mixtures. The equilibrium is far toward the formation of acetyl phosphate and formate so that a very small amount of pyruvate is formed. However, considered in view of proof provided by the heavy carbon experiments it seems quite probable that the measured compound is really pyruvate. According to these measurements the equilibrium constant is roughly 10^{-2} for the condensation reaction.

The exchange of radioactive inorganic phosphate with the phosphorus of acetyl phosphate was also determined. The equilibrium between the organic and inorganic phosphate was reached in about one-half hour. Lipmann and Tuttle have concluded that the phosphate exchange is caused predominantly by Reaction 6 although the possibility of other reactions occurring is considered. It is to be noted that the rate of exchange was not influenced by addition of any reactant other than acetyl phosphate. This is surprising if the exchange occurs by the phosphoroclastic reaction.

Closely related to the above investigations are the experiments by Koepsell

and Johnson (38) and Koepsell *et al.* (39). They obtained a cell-free extract from *Clostridium butylicum*, which catalyzed the fermentation of pyruvate to acetate, carbon dioxide and hydrogen. The amount of hydrogen evolution during the reaction was proportional to the concentration of inorganic phosphate, but with ordinary methods for phosphate determination no phosphate ester could be detected (38). However, by using a procedure applicable to the determination of labile phosphate esters, an ester could be separated which quite certainly was acetyl phosphate in mixture with butyryl phosphate (39). The butyryl phosphate apparently is formed by transfer of the phosphate of acetyl phosphate to butyric acid.

Reaction 10 which is catalyzed by the extract of the butyl bacteria is thus almost identical with that by the extract from *E. coli*.

(10) $CH_3 \cdot CO \cdot COOH + H_3PO_4 \rightleftharpoons CH_3 \cdot CO(OPO_3H_2) + CO_2 + H_2$

The difference in the reactions is that formate is not a product and apparently not an intermediate since formate is not broken down by the enzyme from butyl bacteria. Lipmann and Tuttle (16) have provided evidence for the view that Reaction 10 is reversible. They showed that about the same amount of pyruvate is formed by this enzyme from acetyl phosphate and carbon dioxide and hydrogen as was observed with the extract from *E. coli* and acetyl phosphate and formate. Very recently Wilson, Krampitz and Werkman (40) have added to the evidence by experiments with $C^{13}O_2$. Using a cell-free extract of *Cl. butylicum* with pyruvate, $C^{13}O_2$ was found to exchange rapidly with the carboxyl group of pyruvate. It is thus clear that carbon dioxide may be fixed by C_2 and C_1 addition by two different bacteria (intact *E. coli* cells can reduce carbon dioxide with hydrogen to formate). Furthermore, it is reasonably certain that acetyl phosphate is an intermediate in the reaction.

Whether or not acetyl phosphate plays a rôle in animal tissue is not known at present. The comparative biochemistry of different tissues has thus far proved similarity to be the rule and it seems probable that animal tissue will be found to produce acetyl phosphate. Lipmann (41) has recently described an enzyme occurring in skeletal muscle which rapidly and specifically dephosphorylates acetyl phosphate. Its presence in tissue might readily interfere with the detection of any acetyl phosphate which was present. The establishment of acetyl phosphate as an intermediate in animal metabolism will not necessarily imply that animals can fix carbon dioxide by C_2 and C_1 condensation. It is to be noted that there are no reactions known to occur in animals which are comparable to the reactions which are reversible in bacteria, i.e., reactions in which formate or hydrogen gas is produced. If the dismutation of pyruvate to lactate and acetate and carbon dioxide were found to be reversible, the possibility of fixation of carbon dioxide by C_2 and C_1 addition in animals would seem much more probable, since this conversion apparently occurs in animal tissue (42, p. 210).

Although carbon dioxide fixation in the carboxyl group of lactic acid, as shown by Wood *et al.* (11), (24) with pigeon liver and with the liver extract may possibly occur by C_2 and C_1 condensation, the fixation seems to be adequately accounted

for by C_3 and C_1 condensation. The liver extract apparently does not break down pyruvate, therefore the possibility of C_2 and C_1 addition in this case seems remote.

C_4 and C_1 addition by oxalosuccinate carboxylase. Ochoa and Weisz-Tabori (43) have recently made the very interesting discovery that crude solutions of isocitric dehydrogenase prepared from heart muscle contain an enzyme which catalyzes Reaction 11.

(11) $\mathrm{COOH \cdot CH_2 \cdot CH(COOH) \cdot CO \cdot COOH} \rightleftharpoons$

Oxalosuccinic acid

$$\mathrm{COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH} + \mathrm{CO_2}$$

α-Ketoglutaric acid

Chemical synthesis of oxalosuccinic acid was accomplished for the first time and the decarboxylation of the acid was then studied with the enzyme preparation. The enzyme is thermolabile and is activated by Mn^{++} but not by Mg^{++}. It can be precipitated with acetone from aqueous solution without loss of activity and is almost completely precipitated by acidification to pH 5.2. The enzyme does not catalyze the decarboxylation of oxalacetic acid.

Previously the decarboxylation of oxalosuccinate had been considered to be a spontaneous, non-enzymatic reaction (44). However, Evans (1) had suggested that the decarboxylation might be enzymatic, cf. Moulder *et al.* (138) for the complete account of the investigation. This suggestion was based on observations made with pigeon liver extract in studies of Reaction 12.

(12) pyruvate + citrate → α-ketoglutarate + carbon dioxide + lactate

While the pigeon liver extract catalyzed this reaction, similar extracts from pigeon breast muscle did not, although the requisite enzymes, lactic and isocitric dehydrogenases together with coenzymes, were present. However, when a small amount of purified pigeon liver extract was added to the muscle system the reaction proceeded. Moulder *et al.* suggest that the liver preparation might serve in the decarboxylation of oxalosuccinate, and that this would explain the necessity of Mn^{++} or Mg^{++} ions for isocitrate oxidation. These ions were considered unusual cofactors for the pyridine nucleotide enzymes, lactic and isocitric dehydrogenases.

These authors (138) also pointed out the interesting fact that Reaction 12 involves the interaction of a lactate-pyruvate system which is thought to be coenzyme I specific with an isocitrate-α-ketoglutarate system which is thought to be coenzyme II specific. They raise the question of whether or not these enzymes may not react with both cofactors.

Not only has a specific enzyme for oxalosuccinate been demonstrated (43) but Ochoa (19) has shown in addition that the reaction is reversible, i.e., that carbon dioxide combines with α-ketoglutarate to form oxalosuccinate. Since labeled carbon dioxide was not used it was necessary to remove the oxalosuccinate by

reduction to isocitrate (Reaction 13), in order to shift the reaction toward carbon dioxide fixation. Reaction 13 was followed by determining spectrometrically the $TPN_{ox.}$[2]

(13) $COOH \cdot CH_2 \cdot \underset{\displaystyle COOH}{\underset{|}{CH}} \cdot CO \cdot COOH + TPN_{red.} \rightleftharpoons$

Oxalosuccinic acid

$$COOH \cdot CH_2 \cdot \underset{\displaystyle COOH}{\underset{|}{CH}} \cdot CHOH \cdot COOH + TPN_{ox.}$$

Isocitric acid

In the presence of the heart enzyme the oxalosuccinate for Reaction 13 can be replaced by α-ketoglutarate and carbon dioxide. The α-ketoglutarate and carbon dioxide combine according to Reaction 11 and the resulting oxalosuccinate is reduced to isocitrate. The reaction can be shifted still further toward carbon dioxide fixation by combining it with a glucose-6-phosphate dehydrogenase system. TPN_{red} is supplied by the glucose-6-phosphate dehydrogenase system (Reaction 14) for reduction of the oxalosuccinate.

(14) glucose-6-phosphate + $TPN_{ox.} \rightarrow$ 6 phosphogluconate + TPN_{red}

The net result of the combined systems is the following dismutation:

(14a) α-ketoglutarate + CO_2 + glucose-6-phosphate = isocitrate + 6 phosphogluconate

The isocitrate was determined with isocitric dehydrogenase.

The evidence is thus clear that carbon dioxide combines with α-ketoglutarate to form oxalosuccinate. Since oxalosuccinate and oxalacetate are both β-keto acids and the carboxylation in both cases occurs β to the keto group, the reactions have much in common. It is possible that further study may reveal that ATP is a component of the oxalosuccinate carboxylase reaction, as it is in the oxalacetate carboxylase reaction. It is to be noted that both reactions are simple carboxylations whereas the reaction of acetyl phosphate with carbon dioxide involves a simultaneous reduction.

Ochoa has pointed out that the equilibrium of the isocitrate system in the presence of aconitase would be shifted toward carboxylation of α-ketoglutarate, because over ninety per cent of the isocitric acid would be removed to form cis-aconitate and citrate. The aconitase-isocitric dehydrogenase-oxalosuccinate carboxylase system could then play an important part in the biological utilization of carbon dioxide.

In the Krebs cycle it is usual to consider the reactions as oxidative processes in which the DPN_{red} is kept at a low concentration and the reactions are shifted toward decarboxylation. It is evident, however, since the reactions are reversible, that under suitable conditions the reactions may be shifted toward syn-

[2] TPN = triphosphopyridine nucleotide.

thesis. These reactions may then serve in synthesis to supply needed components for maintenance and biochemical reactions of the animal. Furthermore, the reactions we have just considered pass in steps from C_2 (acetate), to C_3 (pyruvate) to C_4 (oxalacetate), then miss a step C_4 to C_5 and then go from C_5 (α-ketoglutarate), to C_6 (isocitrate). If the conversion of succinate to α-ketoglutarate were shown to be reversible, a model would then be available for reversal of the Krebs cycle, in which isocitrate would be synthesized from a C_2 compound and 4 carbon dioxide molecules. It is clear that reactions gradually are being derived from studies with animals and heterotrophic bacteria, which may eventually help to explain the chemistry of photosynthesis.

SUMMARY. Three initial reactions for the fixation of carbon dioxide have been demonstrated.

Carboxylation by the oxalacetate β-carboxylase reaction was first demonstrated with bacteria and has now been shown conclusively with Evans' liver preparation. ATP is an essential component of the reaction. It is probable that a high energy derivative of oxalacetate or pyruvate is formed with which the carboxylation occurs.

Fixation of carbon dioxide by C_2 and C_1 addition has been demonstrated in bacteria. The C_2 component is almost certainly acetyl phosphate, which condenses with formic acid or carbon dioxide and hydrogen. A corresponding reaction in animal tissue has not been demonstrated as yet.

The carboxylation of α-ketoglutarate to yield oxalosuccinate has recently been demonstrated with a specific enzyme from heart muscle. There is some evidence of a similar enzyme in liver.

Except for one carboxylation, the synthesis of a C_6 compound from acetic acid and four molecules of carbon dioxide can now be pictured with reversible enzyme reactions. It is obvious that these studies may help to explain the chemistry of photosynthesis.

SECTION II. MECHANISM OF FIXATION OF CARBON DIOXIDE IN DIFFERENT COMPOUNDS. Fixation of carbon dioxide has been shown to occur in some 17 different compounds and probably occurs in others. For a number of these compounds the mechanism of fixation has not been clarified but there is no reason to believe the reactions may not be of considerable biochemical importance. For others there is a fairly clear concept of the reactions which, for the most part, are largely based on the same fundamental mechanisms. This subject has been considered in a previous review (2) and will be summarized in detail in this communication only in relation to the more recent developments. The compounds will be considered in the order of increasing length of the carbon chain and since there have been several recent investigations on the fixation of carbon dioxide in acetate and glycogen, these compounds will be discussed in some detail.

C_1 compounds. Methane. The reduction of carbon dioxide to methane has been demonstrated with labeled carbon by Barker *et al.* (45) with methane bacteria. According to Barker's views (46), all types of methane fermentations by bacteria may be considered as oxidations in which carbon dioxide acts as the hydrogen acceptor.

(15) $$4H_2A + CO_2 \rightarrow 4A + 2H_2O + CH_4$$

H_2A is an oxidizable molecule and A is the oxidation product.

The intermediate compounds of the reduction of carbon dioxide to methane are not known (Barker (47)).

Urea. The synthesis of urea by the ornithine cycle as proposed by Krebs and Henseleit (48) involves utilization of carbon dioxide. Proof that carbon dioxide is fixed in urea by liver has been shown with $C^{13}O_2$ (49) and $C^{11}O_2$ (50).

Formic acid. Woods in 1936 (51) demonstrated that carbon dioxide can be reduced by gaseous hydrogen to formic acid with *E. coli.* This reaction, which is catalyzed by hydrogenlyase, probably occurs with other bacteria. In fermentations of glucose by *Aerobacter indologenes* (5) and by *Cl. butylicum* (30) and in the dissimilation of pyruvate by *E. coli* (52), (18), $C^{13}O_2$ has been shown to be converted to formate. Since formate can be produced from pyruvate directly without exchange of carbon dioxide with the formate (53) it is possible in some of these fermentations that the C^{13}-formate was formed from intermediary C^{13}-pyruvate. However, the above named organisms contain hydrogenlyase and the C^{13}-formate sometimes contained more C^{13} than the carboxyls of the pyruvate or lactate, so it is reasonably certain that some of the C^{13}-formate arose by direct reduction.

C_2 compounds. Acetic acid. Carbon dioxide is fixed in the carboxyl group of acetic acid arising in glucose fermentations by *Aerobacter indologenes* and by *Cl. welchii* (5). A different type of fixation occurs in the fermentations of uric acid by *Cl. acidi-urici* (6). In this case both positions of the produced acetic acid contained fixed carbon and sixty-seven per cent of the radio-activity was in the methyl group and thirty-three per cent in the carboxyl group.

Another organism which converts carbon dioxide to acetic acid is *Cl. thermoaceticum.* It ferments glucose (54) and pyruvate or xylose (55) with the formation of more than two moles of acetate per mole of carbohydrate and more than one mole per mole of pyruvate. This is more acetate than can arise by the ordinary C_2 and C_1 type of cleavage through which fermentations have generally been considered to occur. Barker and Kamen (134) have very recently demonstrated with this organism that carbon dioxide is fixed in both positions of acetate, when glucose is fermented in the presence of C^{14}-Na_2CO_3. The glucose was found quantitatively converted to acetate, $C_6H_{12}O_6 = 3CH_3COOH$. The authors present evidence that the glucose is dissimilated as follows:

$$C_6H_{12}O_6 + 2H_2O = 2CH_3 \cdot COOH + 2CO_2 + 8H$$

and that the resulting CO_2 is reduced to acetate

$$8H + 2CO_2 = CH_3COOH + 2H_2O.$$

Barker *et al.* (135) have also found that *Butyribacterium rettgeri* fixes carbon dioxide during the fermentation of lactic acid to acetic and butyric acids. In the presence of $C^{14}O_2$ both carbons of the acetic acid contained isotope as well as all carbons of the butyric acid. It is suggested that acetic acid is formed by condensation of carbon dioxide and evidence is presented that the butyric acid is

formed by condensation of acetic acid. These fixations which occur in more than one position of the acetate involve apparently, though not necessarily, a carbon to carbon linkage in which both components are formed from carbon dioxide. It is possible that part of the acetate molecules might have fixation in the methyl group alone and another part of the molecules in the carboxyl group. The analysis would not detect the difference between this mixture and a material in which both the carbons of acetate contained isotope. However, Weiringa (136) has shown that another species of Clostridium, *Cl. aceticum*, can reduce carbon dioxide to acetate with hydrogen gas in a medium containing an extract of mud. In this case, apparently both carbons in the acetate are formed from inorganic carbon. It therefore seems reasonable that other species of Clostridium may bring about such a synthesis.

A mechanism for these fixations has not been suggested but they are of special interest because of the similarity to autotrophic synthesis. The mechanism may be by a cycle involving repeated addition of carbon dioxide to an organic substance, following which acetate is split off. This cycle may involve some of the primary fixation reactions described in Section I; for example, a reversal of the tricarboxylic acid cycle could give such an acetate.

Slade and Werkman (56) have studied in some detail the mechanism of formation of acetate by *Aerobacter* in which carbon is fixed exclusively in the carboxyl group. They suggest that acetate may be formed from succinate by *Aerobacter* according to Reaction 16. The actual components of the reaction may be derivatives of acetate and succinate.

(16) $$C^{13}OOH \cdot CH_2 \cdot CH_2 \cdot C^{13}OOH + 2H \leftrightharpoons 2CH_3 \cdot C^{13}OOH$$

Evidence of Reaction 16 was obtained by adding $CH_3 \cdot C^{13}OOH$, $C^{13}H_3 \cdot C^{13}OOH$, and $C^{13}OOH \cdot CH_2 \cdot CH_2 \cdot C^{13}OOH$ to glucose fermentations. The succinate formed in the fermentations with $CH_3 \cdot C^{13}OOH$ contained C^{13} exclusively in the carboxyl groups while that formed with $C^{13}H_3 \cdot C^{13}OOH$ contained equal concentrations of C^{13} in the methylene and carboxyl carbons. From the succinate, acetate with the C^{13} only in the carboxyl group was formed.

These conversions could not have resulted from carbon dioxide fixation after oxidation of the C^{13}-compounds to carbon dioxide, first, because the C^{13} concentration of the carbon dioxide was lower than that in the acetate or succinate which was formed, and second, because by carbon dioxide fixation the C^{13} would only be in the carboxyl group (5) whereas, when doubly labeled acetate was used, C^{13} was found to occur equally in all positions of the succinate. It also is apparent that the $CH_3C^{13}OOH$ was not converted extensively to acetyl phosphate and then to pyruvate by the phosphoroclastic reaction, for in such a case it would be expected that succinate with C^{13} in the methylene groups would have resulted by the oxalacetate β-carboxylase fixation and reduction of the resulting oxalacetate.

(17) $$CH_3 \cdot C^{13}O(OPO_3H_2) + HCOOH \leftrightharpoons CH_3 \cdot C^{13}O \cdot COOH + H_3PO_4$$

(18) $$CO_2 + CH_3 \cdot C^{13}O \cdot COOH \leftrightharpoons COOH \cdot CH_2 \cdot C^{13}O \cdot COOH \leftrightharpoons COOH \cdot CH_2 \cdot C^{13}H_2 \cdot COOH$$

Kalnitsky *et al.* (53) have obtained somewhat similar results with a cell-free enzyme preparation from *E. coli*. When pyruvate was fermented by this preparation in the presence of $CH_3C^{13}OOH$, succinate was formed which contained C^{13} in the carboxyl groups. Since there was no excess C^{13} in the carbon dioxide produced in the dissimilation it is clear that the C^{13}-succinate was not formed by fixation of carbon dioxide. It appears that this enzyme preparation offers a promising opportunity to study this interesting reaction.

Slade and Werkman consider their results to constitute conclusive proof for Reaction 16. While their evidence for Reaction 16 is quite convincing, it nevertheless is possible that the changes observed result by some other mechanism. For example, in the tricarboxylic acid cycle (fig. 1, p. 212) succinate with labeled carbon in the carboxyl group might be formed from carboxy labeled acetate by Steps[3] 19, 5, 6, 7, 8, 10. Also from the doubly labeled acetate, succinate with the isotope in both the methylene and carboxyl groups would result. In addition, by the reversal of these steps labeled acetate could be formed from succinate. At the present time there is no evidence for the reversibility of Step 10 of the cycle, i.e., succinate to α-ketoglutarate, but likewise there is no direct evidence with an isolated enzyme system plus succinate and acetate that Reaction 16 as such is reversible.

The problem is of considerable importance, for it raises again the question of whether or not oxidative conversion of pyruvate to carbon dioxide may occur according to the older proposal of Thunberg (57). This proposal has for some time been dropped from consideration largely because of the lack of evidence for Reaction 16. This cycle as shown below starts with two molecules of pyruvate; one is again regenerated and three molecules of carbon dioxide are formed.

$$\begin{array}{ccc} 2CH_3\cdot CO\cdot COOH & \longrightarrow & 2CH_3\cdot COOH + 2CO_2 \\ \uparrow & & \downarrow \\ \uparrow -CO_2 & & \downarrow \\ COOH\cdot CH_2\cdot CO\cdot COOH & \longleftarrow & COOH\cdot CH_2\cdot CH_2\cdot COOH \end{array}$$

With animal tissue there has been no evidence of fixation of carbon dioxide in acetate and as yet no comparable proof for Reaction 16.

The earlier results of Sonderhoff and Thomas (108) should be considered in this connection. These results were obtained by isolating succinate and citrate from the products of the oxidation of $CD_3\cdot COOH$ by yeast. The concentration of the deuterium in the succinate and citrate was then determined. Since the four methylene hydrogens of the six hydrogen linkages of succinate are stable and not subject to exchange with hydrogen, the ratio of deuterium to hydrogen plus deuterium would be expected to be 0.67 (4/6), if succinate was formed by direct condensation of $CD_3\cdot COOH$. Likewise for citrate in which four out of eight of

[3] The individual conversions of figure 1 have been numbered and are referred to as *Step* 1, etc.; on the other hand, the conversions which have been illustrated in the text proper are designated as *Reaction* 1, etc.

the hydrogens are stable, this ratio would be 0.50. The observed ratios were 0.27 (40 per cent of the possible 4D) for succinate and 0.28 (56 per cent of the possible 4D) for citrate. On the assumption that there was some dilution of the succinate by endogenous reactions it seemed probable that approximately 2D from the acetate were incorporated in the succinate and citrate.

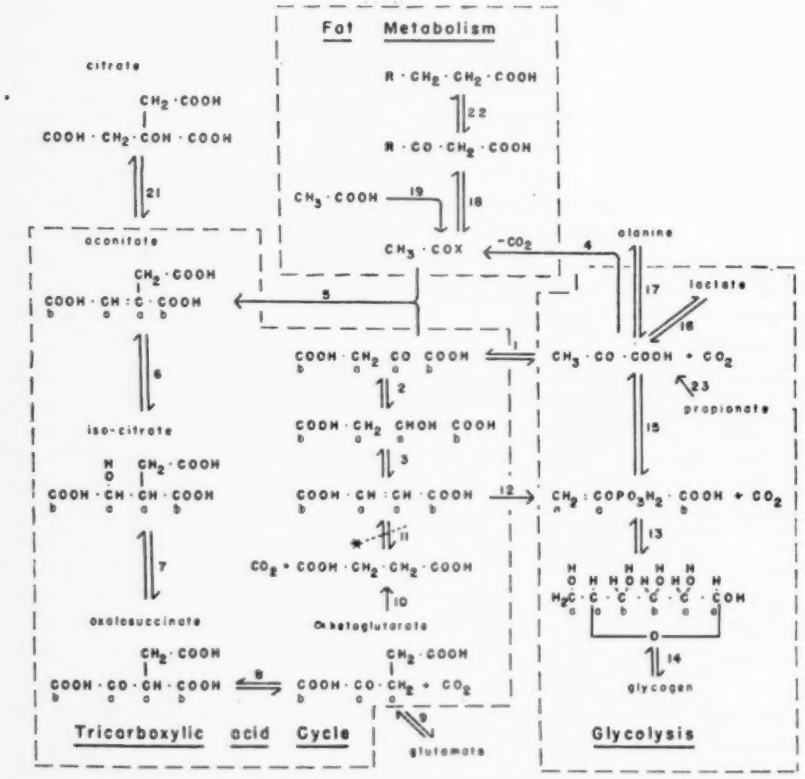


Fig. 1. Interrelationships of the tricarboxylic acid cycle and carbohydrate, fat, and protein metabolism.

* Malonate inhibition.

Lynen (87) has pointed out that the succinate might be formed via the tricarboxylic acid cycle, figure 1. However, he concludes that the symmetrical citrate molecule could not be an intermediate for if it were, the succinate would contain only 1D. His point is clear if one traces the hydrogen atoms on the methyl of the acetyl compound of figure 1 through Steps 5, 6, 7, 8, 10. In all of these steps the hydrogen of the original methyl group is stable and the result-

ing succinate would therefore contain 2D. However, if a direct conversion of oxalacetate and acetate occurred to give citrate with the following formula:

$$\begin{array}{l} CD_2 \cdot COOH \\ | \\ COOH \cdot CH_2 \cdot COD \cdot COOH \end{array}$$

then upon conversion of citrate to aconitate (Step 21) the deuterium on the carbinol group would be lost. Furthermore, the deuterium labeled methylene group and normal methylene group would be converted equally to the carbonyl in oxalosuccinate. Thus, one half of the deuterium of the methylene groups of citrate would be lost, therefore, there would be only 1D in the succinate formed from the citrate.

There is one point, however, that is not accounted for in these considerations, i.e., how there can be a net production of citrate and succinate via the cycle with acetate as the only substrate. The tricarboxylic acid cycle does not provide for this fact. In the cycle a C_4 dicarboxylic acid is required for the original condensation (Step 5) and only by the completion of the cycle is the C_4 acid regenerated. It thus becomes necessary to assume that enough C_4 dicarboxylic acids were produced in Sonderhoff and Thomas' experiments to provide for the observed accumulation of citrate and succinate. It seems questionable whether the endogenous respiration could be the source of this quantity of C_4 acids.

Actually the experimental results may be consistent with a mechanism involving formation of succinate by direct condensation of acetate via Reaction 16. The succinate formed from $CD_3 \cdot COOH$ by this reaction would contain four D but the ultimate concentration of D in the succinate of the reaction mixture would depend on the relative rates of several reactions. For example, by reversible conversion of succinate to fumarate, Step 11, the D from the succinate could be rapidly lost. Also succinate formed via Step 10 would influence the D content of the succinate. It is clear that a reliable prediction of the D content of the succinate cannot be made unless the relative rates of the reactions are known.

Furthermore, in the conversion of succinate to oxalacetate by Steps 11, 3, and 2, three of the hydrogens of the methylene groups of the succinate are removed and in addition the fourth hydrogen would in part be lost via formation of the enol of oxalacetate. It is thus evident that even though the C_4 dicarboxylic acid were formed from trideuteroacetate by direct condensation, the citrate formed via Steps 5 and 21 would not be expected to contain much more than 2D. It is apparent therefore that the results of Sonderhoff and Thomas (108) are not in disagreement with formation of succinate by direct condensation of acetate. Obviously deuterio labeled acetate is not a satisfactory compound for study of this particular problem. Further investigation is needed, before the reactions can be defined by which yeast forms citrate and succinate from acetate.

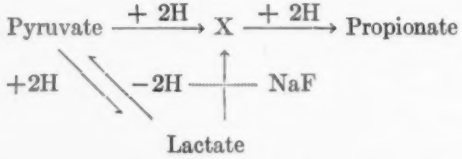
C_3 compounds. Pyruvic acid. The fixation of carbon dioxide in pyruvate has been demonstrated with *E. coli* (18) *Cl. butylicum* (40), and with pigeon liver extract (24). The mechanism of these fixations is by the phosphoroclastic re-

action with the bacterial preparations and probably by oxalacetate β-carboxylase with pigeon liver, cf. Section I, p. 205.

Lactic acid. Carbon dioxide fixation in lactate has been demonstrated with a large number of species of bacteria (5), (30) and with pigeon liver (11), (24). The lactate presumably is derived from pyruvate in which fixation occurs by the two mechanisms mentioned above.

Propionic acid. Carbon dioxide is fixed in propionate during fermentations of glycerol, glucose or pyruvate by propionic acid bacteria (58), (52). The fixed carbon is exclusively in the carboxyl group of the propionate (59), (60). The mechanism of propionate formation is not known but from a consideration of the amount of carbon dioxide fixed in the propionate and succinate, it seems probable that the propionate is derived from a symmetrical dicarboxylic acid in which only one of the carboxyls is from fixed carbon dioxide. This derivation accounts for the fact that in glycerol fermentations the propionate carboxyl contains roughly one-half as much excess C^{13} as the carbon dioxide and the same per cent as the average of the succinate carboxyls (52), (2). It is necessary in this scheme to assume that the rates of the reactions are rapid toward succinate and propionate, otherwise by reversal of the reactions both carboxyls of the symmetrical acid would be in equilibrium with the $C^{13}O_2$.

Related to this problem are the results of Barker and Lipmann (61) with the propionic acid bacteria. They found, in confirmation of Chaix-Audermard (62), that sodium fluoride inhibits the conversion of lactate to propionate but not of pyruvate to propionate. In studying the details of the inhibition it was found that the reduction of lactate but not the oxidation of lactate was inhibited by the fluoride. Moreover, lactate did not accumulate in the fermentation of pyruvate in the presence of fluoride. This observation was considered as evidence that laluote is not an intermediate in the reduction of pyruvate to propionate. The artəlwing scheme was suggested by Barker and Lipmann to account for their clotsfs.



The hypothetical intermediate compound X is not believed to be acrylic acid. It is possible, in view of the C^{13} experiments, that X is a dicarboxylic acid which is formed by the fixation reaction.

Propyl alcohol. Propyl alcohol containing fixed carbon is formed in the fermentation of glycerol (52). It seems likely that it is formed by reduction of propionate which contains fixed carbon.

C_4 compounds. Oxalacetic acid. The fixation of carbon dioxide in oxalacetate has been shown conclusively by Krampitz *et al.* (17) with bacteria and by Utter

and Wood (25) with pigeon liver extract. The reaction is catalyzed by oxalacetate β-carboxylase and is considered in detail in Section I, p. 200.

Succinic acid. Bacteria (58), (52), (5), (53), protozoa (9) and pigeon liver (11) have been shown with labeled carbon to fix carbon dioxide in succinate during the dissimilation of a variety of substrates. It is interesting that the fixation studied by Kalnitsky *et al.* (53) was obtained with a cell-free preparation. Prior to the above investigation with protozoa (9) Searle and Reiner (8) had shown that the protozoan, *Trypanosoma lewisi*, could cause a net uptake of carbon dioxide in glucose dissimilations. Labeled carbon dioxide was not used in the investigation, therefore the fixed carbon could not be located but it very likely was fixed in succinate, since the carbon dioxide assimilated was equivalent to the succinate formed.

All investigators have concluded that the fixation of carbon dioxide in succinate is by the oxalacetate carboxylase Reaction 1 and by subsequent reduction of the oxalacetate. In every case of fixed carbon dioxide in succinate the fixed carbon has been found to be exclusively in the carboxyl groups. It perhaps should be mentioned that in view of Slade and Werkman's (56) observation that acetic acid may condense to succinate (Reaction 16), it is conceivable that carbon dioxide could be fixed in acetate and then converted to succinate. Succinate with fixed carbon might be formed in this case independent of C_3 and C_1 addition.

Fumaric and malic acids. Fumarate and malate are considered intermediates in the conversion of oxalacetate to succinate and it is to be expected that these compounds would contain fixed carbon. Nishina *et al.* (63) with radioactive carbon have demonstrated the synthesis of malate and fumarate from pyruvate and carbon dioxide in fermentations by *E. coli*. Also, Foster *et al.* (7) with the mold *Rhizopus nigricans* have shown that carbon dioxide is fixed exclusively in the carboxyl groups of fumarate. Similarly, in pyruvate dissimilations by pigeon liver (11), (24), the fixed carbon is in the carboxyl groups of the malate and fumarate.

Butyric acid. Carbon dioxide is fixed in butyrate by *Butyribacterium rettgeri* (135) (cf. acetate above).

C_5 compounds. α-Ketoglutaric acid. The first proof of carbon dioxide fixation in a carbon to carbon linkage by animal tissue was Evans and Slotin's (64) demonstration that pigeon liver when acting on pyruvate synthesizes α-ketoglutarate containing carbon from $C^{11}O_2$. It was shown by Wood *et al.* (65), (11) and independently by Evans and Slotin (66) that the fixed carbon was exclusively in the carboxyl alpha to the keto groups of this dicarboxylic acid. The statement is incorrect (67) that Evans and Slotin proved this location of the fixed carbon in their first paper (64); no discussion concerning the location of the fixed carbon or of a degradation of the α-ketoglutarate occurs in this report.

Thus far there has been no proof of fixation of carbon dioxide in α-ketoglutarate by molds, yeast, protozoa or bacteria; probably because the compound is difficult to isolate in dissimilations by these organisms.

The mechanism of fixation of carbon dioxide in α-ketoglutarate will be considered in Section III, p. 221, under the tricarboxylic acid cycle.

C_6 compounds. Oxalosuccinic acid. At present there is but one report (19) concerning fixation in this compound and it has been considered in detail in Section I.

Citric acid. Neither isocitrate nor citrate has been isolated as yet from pigeon liver experiments in which labeled carbon dioxide was used. Thus, there is no direct proof of fixation in these compounds by pigeon liver, though such is to be expected according to the tricarboxylic acid cycle. In the experiments of Ochoa (19) with heart muscle preparation, α-ketoglutarate was converted to isocitrate and the isocitrate therefore contained fixed carbon. This experiment was done with non-labeled carbon dioxide.

The only direct proof of fixation of carbon dioxide in citrate has been provided by Foster *et al.* (7) with the mold *Aspergillus niger*. They have shown that citric acid as formed in the fermentation of sucrose contains fixed carbon which apparently is restricted to the carboxyl groups. It is suggested that the mechanism may be that of the Krebs cycle.

Glycogen. The group of investigators at Harvard has contributed very interesting information on the fixation of carbon dioxide (12), (68), (69) and the conversion of lactate (70), (71) and lower fatty acids (72) (acetate, propionate, butyrate) to glycogen. These experiments are especially significant because they were the first in which the *in vivo* fixation of carbon dioxide was studied with normal animals. Likewise, the experiments of Lorber *et al.* (13) with the isolated working heart give evidence that fixation of carbon dioxide in glycogen is a normal process in this organ.

The procedure followed in the Harvard fixation experiments was to fast the rats for 24 hours, then to feed 150 mgm. of lactate (12) or 300 to 600 mgm. of glucose (68) by stomach tube and to administer radioactive bicarbonate intraperitoneally in five doses at half hour intervals. The glycogen was isolated from the liver at the end of the 2.5 hour period.

The results were quite similar after either lactate or glucose feeding. The glycogen contained 0.3 to 1.5 per cent of the administered radioactive C^{11} and 45 to 75 per cent of the C^{11} was eliminated in the expired air. An average of 11 per cent of the liver glycogen was calculated to have been derived from fixed carbon dioxide with lactate and 13 per cent with glucose. In the muscle glycogen after lactate feeding there was no significant incorporation of C^{11}. With glucose there was an appreciable amount, the radioactivity on comparable quantities of glycogen being 10 to 30 per cent of that of the liver glycogen.

The mechanism proposed by Solomon *et al.* (12) for the conversion of pyruvate, lactate, etc., to glycogen has been of special interest because it assigned to the fixation reaction an essential function in glycogen synthesis. This function was to circumvent the only step believed irreversible in glycolysis, i.e., the conversion of pyruvate to phosphopyruvate (73). This reaction has now been shown to be reversible by Lardy and Ziegler (74) so that the fixation reaction can no longer be considered obligatory on this basis. It is still conceivable, of course, that the fixation may in part at least supply the phosphopyruvate for the synthesis.

The mechanism proposed by Solomon *et al.* for the fixation of carbon dioxide in glycogen was as follows:

(19) $C^*O_2 + CH_3 \cdot CO \cdot COOH \leftrightharpoons C^*OOH \cdot CH_2 \cdot CO \cdot COOH$

(20) $C^*OOH \cdot CH_2 \cdot CO \cdot COOH \leftrightharpoons C^*OOH \cdot CH{:}CH \cdot COOH$

(21) $C^*OOH \cdot CH{:}CH \cdot COOH \rightarrow CH_2{:}C(OPO_3H_2) \cdot C^*OOH + CO_2$

or

$$CH_2{:}C(OPO_3H_2) \cdot COOH + C^*O_2$$

The net result of these reactions is that pyruvate is phosphorylated and carbon dioxide is fixed in the carboxyl of the phosphopyruvate. The remainder of the path to glycogen was viewed as the reversal of the current schemes of glycolysis by which two molecules of pyruvate are converted to a molecule of glucose. In this conversion the carboxyls of the pyruvate make up the 3 and 4 positions of the glucose. The Harvard group considered that one out of two of these carbons, on the average, would contain fixed carbon. The fixed carbon in the glycogen, thus, should be 16.7 per cent (one in six). This value was approached in the Harvard experiments, on the basis of calculations which included certain assumptions. The finding of approximately this amount of fixed carbon in the glycogen was considered the principal evidence for the scheme. However, if the rates of Reactions 19 and 20 were sufficiently rapid to the left, both carboxyls of fumarate would be in equilibrium with the carbon dioxide, and in this case fixed carbon would be in all molecules of the pyruvate. The fixed carbon in the glycogen would then be 33.3 per cent. It seems possible that the observation of approximately 16 per cent fixation may have resulted from fortuitous circumstances.

Wood *et al.* (75) have recently conducted some further studies on the fixation of carbon dioxide in glycogen. They determined the position of the fixed carbon in the glucose unit of the glycogen. The general procedure of treatment of the animals was similar to that of the Harvard experiments, except that C^{13}-bicarbonate was used. The liver glycogen was isolated and then converted to glucose which was then degraded so that the position of the heavy carbon in the glucose could be determined.

Two different types of degradation were used. In the first the glucose was fermented to lactate by lactic acid bacteria. The lactate was then oxidized with permanganate to acetaldehyde and carbon dioxide, and the acetaldehyde was further degraded to iodoform and formic acid. As indicated in the equations, the carbon dioxide is from positions 3 and 4 of the glucose, the iodoform from 1 and 6, and the formic acid from 2 and 5.

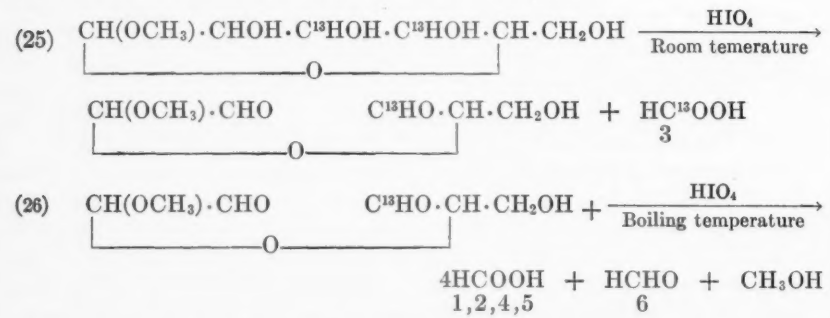
(22) $CHOH \cdot CHOH \cdot C^{13}HOH \cdot C^{13}HOH \cdot CH \cdot CH_2OH \rightarrow$ (1–O–5, 2–3–4, 6)

$$2CH_3 \cdot CHOH \cdot C^{13}OOH$$

(23) $CH_3 \cdot CHOH \cdot C^{13}OOH \xrightarrow{KMnO_4} CH_3 \cdot CHO + \underset{3,4}{C^{13}O_2}$

(24) $CH_3 \cdot CHO \xrightarrow{NaOI} \underset{1,6}{CHI_3} + \underset{2,5}{HCOOH}$

In the second method of degradation the glucose was converted to methyl-glucoside and the latter was oxidized with periodic acid.



The formic acid from the first oxidation at room temperature is from position 3 and the formaldehyde of the oxidation at boiling temperature is from position 6.

The combined results from these two degradations established that the fixed carbon was in positions 3 and 4 of the glucose and that as nearly as could be judged by the methods, the concentration was the same in the two positions. The latter measurement involved technical difficulties which made it subject to considerable error.

Clearly these findings are in full agreement with the scheme proposed by the Harvard Group. However, it is apparent that whether the fixation is essential or not, the distribution of the fixed carbon in the glycogen may be the same. In other words, Reactions 19 and 20 may occur and introduce carbon dioxide in pyruvate but these reactions may have no function in the formation of phosphopyruvate. All of the phosphopyruvate may actually be formed by direct phosphorylation with adenosine triphosphate as shown by Lardy and Ziegler (74).

It should be noted that the shift of the hydroxyl in enol-oxalacetate does not occur spontaneously (17) (cf. Section I, p. 201) and this can no longer be considered a mechanism for the formation of the labeled pyruvate from oxalacetate. It also should be borne in mind that the carbon dioxide may be fixed by C_2 and C_1 additions (Section I) and thus give rise to carboxyl labeled pyruvate and a similar type of glycogen.

There is an interesting possibility for checking whether or not the fixation of carbon dioxide in glycogen involves a symmetrical dicarboxylic acid. It involves the feeding of a carbonyl or methyl labeled pyruvate. If the carbon dioxide is fixed in pyruvate by Reactions 19 and 20, then from these types of pyruvate would be formed a pyruvate with labeled carbon in both the methyl and carbonyl positions and this compound would give rise to glucose labeled in positions 1, 2, 5 and 6. On the other hand, if the fixation of carbon dioxide was by direct exchange in the carboxyl of pyruvate, and the carbonyl labeled pyruvate were used, the glucose would be labeled in positions 2 and 5. A result such as this, or one in which the concentration was greater in the 2 and 5 than the 1 and 6 positions, would be direct proof that some pyruvate was converted to glycogen without

passing through a symmetrical molecule. However, even if the results showed that carbon dioxide fixation occurred by formation of a symmetrical molecule it would not prove that the reaction promoted phosphopyruvate formation.

It is noteworthy that the labeled carbon was not randomized between positions 1 and 3, or 4 and 6 of the glucose in either the animal conversion to glycogen or in the bacterial degradation of the glucose. Thus, at no point in glycolysis was there formed a symmetrical molecule such as dihydroxyacetone. This fact is evident, for if such a compound occurred as an intermediate, the orientation of the carboxyl carbon would be lost during the glycolysis and the fixed carbon would no longer be confined to only the 3 and 4 positions. This fact proves that there was no substantial dephosphorylation of dihydroxyacetone phosphate and subsequent fermentation of the unphosphorylated compound nor was there shifting of phosphate.

A problem, which in an indirect way is related to carbon dioxide fixation, is the conversion of lower fatty acids to glycogen. In this conversion the question arises whether the acid is changed directly to glycogen or whether the labeled carbon is incorporated in the glycogen by carbon dioxide fixation after oxidation of the acid. Buchanan *et al.* (72) have investigated this problem with carboxyl labeled acetate, propionate, and butyrate fed in conjunction with glucose. The radioactivity of the liver glycogen and of the expired carbon dioxide was determined, and on the basis of certain assumptions, the radioactivity of the glycogen, which was due to carbon dioxide fixation, was calculated. In the case of propionate and butyrate a considerable proportion of the acids were estimated to have entered the glycogen in a form other than carbon dioxide. On the other hand, with acetate the total radioactivity of the glycogen was accounted for by fixation of the radioactive carbon dioxide derived from the acetate.

This problem has been investigated further by Lorber *et al.* (76). The reasoning followed in their experiments was that any carbon which enters glycogen as carbon dioxide will be in the 3 and 4 positions and if labeled carbon is found in other positions of the glycogen, it establishes that the compound entered in a form other than carbon dioxide. They have conducted experiments following the procedure of Buchanan *et al.* (72) with the following types of acids: $CH_3 \cdot C^{13}OOH$, $C^{13}H_3 \cdot C^{13}OOH$, $CH_3 \cdot CH_2 \cdot C^{13}OOH$ and $CH_3 \cdot CH_2 \cdot CH_2 \cdot C^{13}OOH$. The degradation procedures of Reactions 22 through 26 were used. It was found that with all three of the carboxyl labeled acids the labeled carbon was in positions 3 and 4 of the glucose of the glycogen. On the contrary, with the acetate which was labeled in both positions, the C^{13} was not exclusively in the 3 and 4 positions but apparently was in all the positions of the glucose. This latter result is interpreted by these investigators as conclusive proof that at least *a part of the* acetate molecule enters glycogen in a form other than carbon dioxide. This constitutes the first direct evidence that acetate can be converted to glycogen. With regard to the findings with the three carboxyl labeled acids, the fact that the tagged carbon was only in the 3 and 4 positions was not considered evidence that this carbon entered the glycogen only in the form of carbon dioxide. The calculations of Buchanan *et al.* (72) indicate that at least part of the butyrate

and propionate carboxyls are not deposited as carbon dioxide. This fact, together with the direct demonstration with the doubly labeled acetate (76) that acetate is converted to glycogen, makes it seem probable that propionate and butyrate may likewise be transformed to glycogen by a mechanism in addition to that of carbon dioxide fixation. However, a more direct proof is to be desired.

A consideration of the mechanism by which these acids are converted to glycogen will be presented in the next section, p. 229, which deals with the tricarboxylic acid cycle.

With regard to the calculation by Buchanan *et al.* (72) of the amount of carbon dioxide fixed, it seems probable that whether or not a carboxyl labeled compound will be estimated to enter entirely by carbon dioxide fixation will depend on the rate of conversion of the compound to glycogen. For example, if carboxyl labeled pyruvate was fed and the rates of Reactions 19 and 20 were rapid as compared to the rates of conversion of pyruvate to glycogen, the carboxyl of the pyruvate would come to equilibrium with the carbon dioxide and the calculations would indicate all of the pyruvate entered by carbon dioxide utilization. Thus, with different carboxyl labeled compounds the proportion, which the calculation would give as entering by carbon dioxide fixation, would depend, in part at least, on the extent conditions were established so that there was little equilibration of the carboxyls with carbon dioxide.

Unidentified compounds. In the experiments by Solomon *et al.* (12) only 61 per cent of the $NaHC^{11}O_3$ injected in rats was accounted for in expired $C^{11}O_2$, unabsorbed $NaHC^{11}O_3$, urine, bone, liver glycogen and body fluids. The soft tissue could not be analyzed and the investigators believe that a large portion of the injected C^{11} was present in it in organic combination. The results of Swenseid (77) are at variance with these results. She could find no evidence of such a substantial fixation of carbon dioxide in mice.

Evans and Slotin (10) found in their experiments with pigeon liver acting on pyruvate that part of the fixed carbon was liberated by chloramine−T. This carbon is believed to have been fixed in the carboxyl groups of amino acids.

Barker *et al.* (45) have obtained evidence that the methane bacteria fix carbon dioxide in protoplasm and Foster *et al.* (7) have obtained similar evidence with molds.

SUMMARY. Carbon dioxide has thus far been shown to be fixed in methane, urea, formate, acetate, pyruvate, lactate, propionate, propyl alcohol, oxalacetate, succinate, fumarate, malate, butyrate α-ketoglutarate, oxalosuccinate, citrate, glycogen and some as yet unidentified compounds. The mechanisms of the fixation of carbon dioxide in these compounds is considered in relation to the three known primary fixation reactions.

The formation of acetate and butyrate with all carbons containing fixed carbon has been demonstrated with bacteria. This finding is of special interest because of its similarity to autotrophic synthesis of compounds from carbon dioxide only.

The fixation of carbon dioxide in the carboxyl group of acetate by *Aerobacter* may take place via reductive cleavage of succinate. Considerable evidence has

been presented indicating there is reversal of this reaction, i.e., synthesis of succinate from acetate.

It has been demonstrated that carbon dioxide is fixed in rat liver glycogen in the 3 and 4 positions of the glucose unit. By application of this finding to acetate metabolism it has been shown that acetate is converted to rat liver glycogen in a form other than as carbon dioxide.

SECTION III. THE TRICARBOXYLIC ACID OR KREBS' CYCLE. The tricarboxylic acid cycle is the only mechanism which has received widespread consideration as an explanation of the chemical reactions involved in biological oxidation yielding carbon dioxide and water. This cycle was first proposed as a result of studies on the oxidation of pyruvate by pigeon breast muscle (78) but has gradually grown in stature until it is now receiving increasing attention as a general mechanism for the oxidation of carbohydrates, fats, and proteins by the animal body. In this broad function of the cycle it is assumed that in part, at least, each of these types of substances may give rise to a common intermediate (e.g., pyruvate or an acetyl group) which is subject to oxidation in the cycle.

The cycle is considered of general occurrence since in modified forms it has been suggested to apply not only to pigeon breast muscle (78), (79) but to liver (80), (81), (2), heart (82), kidney (83), (84), (85), (86) and yeast (87).

Evaluation of the experimental basis of the scheme. The evidence and experimental proof for the tricarboxylic acid or Krebs' cycle has been reviewed by Krebs (42), Evans (1), and Werkman and Wood (2). Therefore only the principal observations which have been used in support of the scheme will be cited and the steps in the cycle where the evidence is considered incomplete will be indicated.

Krebs (42) has pointed out that four observations led to the original hypothesis of the cycle in pigeon breast muscle:

1. The catalytic effect of citrate on respiration.
2. The synthesis of citrate from oxalacetate.
3. The rapid oxidation of citrate, isocitrate, cis-aconitate and α-ketoglutarate.
4. The *oxidative* formation of succinate in the presence of malonate using fumarate or oxalacetate as substrates. This fact proved that there are two mechanisms for formation of succinate since formation by direct reduction via Steps[4] 2, 3, and 11 of figure 1, p. 212, is inhibited by malonate.

The observations listed above occur with both pigeon breast muscle (79) or liver (80). To the above points may be added the following for pigeon liver; they apparently are characteristic of liver and not of muscle because the liver can synthesize dicarboxylic acids by carbon dioxide fixation (10), (11), (81):

5. The synthesis of citrate and α-ketoglutarate from pyruvate (80).
6. The formation of α-ketoglutarate with fixed carbon exclusively in the carboxyl adjacent to the carbonyl group and of succinate containing little

[4] The individual conversions of figure 1 have been numbered and are referred to as Step 1, etc.; on the other hand, the conversions which have been illustrated in the text proper are designated as Reaction 1, etc.

or no fixed carbon from pyruvate and labeled carbon dioxide by pigeon liver homogenate in the presence of malonate (11), (10).

In addition there has recently been added evidence that the cycle functions in the oxidation of acetoacetate by kidney and acetate by yeast. The following are pertinent points from these investigations:

7. Citrate is formed from acetoacetate and oxalacetate by kidney (83), (84), (86), and from acetate and oxalacetate by yeast (87).
8. C^{13}-α-ketoglutarate is formed in oxidations by kidney to which C^{13}-acetoacetate (85) or C^{13}-acetate (85) (88) is added and C^{13}-aspartate and glutamate are formed by the intact animal to which C^{13}-acetate is fed (89). The latter two compounds are important in considerations of the cycle since they are considered to be in biological equilibrium with oxalacetate and α-ketoglutarate respectively.

Whether these observations which have been obtained with different tissues and under different conditions are all the result of a similar cycle, as has been suggested, cannot be definitely answered at present. It is conceivable that the scheme applies only in part to certain tissues and also that tissues have more than one mechanism for oxidation to carbon dioxide.

The oxidation of pyruvate will serve to illustrate the general mechanism of the cycle, figure 1. The pyruvate is oxidized by Step 4 to carbon dioxide and an acetyl group, the acetyl group then unites with oxalacetate in Step 5 to form aconitate. Aconitate is converted to isocitrate by Step 6 and it in turn is oxidized to oxalosuccinate in Step 7. The oxalosuccinate is decarboxylated yielding α-ketoglutarate in Step 8 and this compound is in turn oxidized to succinate in Step 10. The succinate is successively oxidized to fumarate, converted to malate and then oxidized to oxalacetate in Steps 11, 3, and 2. The net result of this series of changes is the oxidation of one molecule of pyruvate to carbon dioxide and water and the regeneration of a molecule of oxalacetate which can again function in the cycle.

The principal modification in the cycle, from its original form, has been to place citrate in a side reaction. It became apparent that citrate was not on the direct path of the cycle, when it was found that α-ketoglutarate, which contained fixed carbon in only one carboxyl group (11), (10), was formed by pigeon liver. This observation excluded citrate as a member of the cycle, because α-ketoglutarate as formed from citrate would contain fixed carbon equally in both carboxyls of the ketoglutarate and not in just one carboxyl. Such a distribution is obligatory from citrate because of the symmetrical nature of the molecule. Likewise, more recent observations have indicated that citrate is not a component of the cycle in oxidation by other tissues. These results will be considered in detail in subsequent discussions of the experiments.

The modified tricarboxylic acid cycle presented in figure 1 satisfactorily meets all the requirements of the eight experimental points which have been listed above and it can be used as a basis for explanation of many other observations on carbohydrate, fat and protein metabolism.

The two experimental observations which have had the greatest weight in the development of the scheme were numbers 4 and 6. These points were based on

the function of malonate as an inhibitor for the reductive formation of succinate from fumarate in Step 11. Krebs has postulated the tricarboxylic cycle via Steps 5, 6, 7, 8 and 10 as the malonate insensitive mechanism of formation of succinate and considers this mechanism part of the normal oxidative process. That the inhibition of malonate is effective was shown in the experiments of Wood *et al.* (11) with pigeon liver acting on pyruvate and $NaHC^{13}O_3$. When malonate was present succinate was formed which contained no excess C^{13} although in the same reaction fumarate and malate occurred containing excess C^{13}. Thus in this case it is apparent that the succinate did not arise from the fumarate. When malonate was not added the dicarboxylic acids all contained about the same amount of C^{13}, indicating that in the absence of the inhibitor the dicarboxylic acids were reversibly interconvertible, the C^{13}-succinate arising by reduction of fumarate, Step 11. There is, therefore, clear evidence of Krebs' contention (79) that there are two mechanisms for formation of succinate, one which is not inhibited by malonate and another which is inhibited.

The origin of α-ketoglutarate via tricarboxylic acids and the evidence of the rôle of the tricarboxylic acids in the cycle has largely been based on the following of the above listed points. Point 1, the catalytic effect of citrate, Point 3, the rapid oxidation of tricarboxylic acids to α-ketoglutarate and succinate, and Points 2 and 5, the synthesis of citrate and α-ketoglutarate from pyruvate or oxalacetate. These results definitely suggest that the tricarboxylic acids may function as intermediates but they do not establish that they are an *essential* part of the mechanism.

The possibility of isocitrate not being on the main path of oxidation but being formed as a side reaction is called to attention by the results of Ochoa (19) who has shown that α-ketoglutarate can be converted by carbon dioxide fixation to isocitrate (cf. Section 1, p. 206). Thus, when α-ketoglutarate accumulates, isocitrate would also tend to accumulate and the formation of tricarboxylic acids can be accounted for. Likewise the rapid oxidation of these compounds can occur by reversal of the reactions studied by Ochoa.

The key to the problem of pyruvate oxidation seems to rest on determining the mechanism of formation of α-ketoglutarate. In the case of pyruvate oxidation by pigeon liver there is an additional fact that comes into consideration, that is, that the α-ketoglutarate contains fixed carbon only in the carboxyl adjacent to the carbonyl group.

A speculative mechanism which would yield α-ketoglutarate with fixed carbon in the proper position would be Reaction 27 involving pyruvate in which $C^{13}O_2$ has been fixed in the carboxyl group.

(27) $CH_3 \cdot COOH + CH_3 \cdot CO \cdot C^{13}OOH \rightarrow COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot C^{13}OOH$

This reaction has been postulated for animal tissue but never proved to occur (90). It is conceivable that a reaction such as this could occur in liver in which heavy carbon pyruvate was formed via the oxalacetate β-carboxylase reaction and acetic acid or some derivative was formed by oxidation of pyruvate or fat. In this scheme Step 5 would not occur and Reaction 27 would serve as a pathway to α-ketoglutarate. The oxidation of pyruvate might then be by conversion of

one molecule of pyruvate to acetate, which would in turn unite with a second molecule of pyruvate and form α-ketoglutarate. The remainder of the oxidation would be Steps 10, 11, 3, 2, 1. The net results would therefore be that from two molecules of pyruvate three molecules of carbon dioxide would be formed and one molecule of pyruvate would be regenerated. If such an oxidation occurred, the requirements of the above listed observations could be accounted for as follows without recourse to tricarboxylic acids as the main path of oxidation.

Points 2, 3, and 5, i.e., formation and rapid utilization of tricarboxylic acids, could take place by the oxalosuccinate carboxylase reaction. Point 6, formation of succinate in the presence of malonate, could be readily explained, since Krebs has shown that oxidation of α-ketoglutarate to succinate is not inhibited by malonate. The resulting succinate would contain no fixed carbon since the fixed carbon of the α-ketoglutarate would be split out in the oxidation. Point 1, the catalytic effect of citrate on respiration might result because of the formation of C_4 dicarboxylic acids from the citrate by Steps 21, 6, 7, 8, 10, etc. The dehydrogenation of malate to oxalacetate is one of the most rapid reactions known for tissue and thus formation of a C_4 dicarboxylic acid might catalyze respiration by providing a hydrogen carrier (cf. Szent-Györgyi (91)).

It is evident that in this scheme whenever citrate or isocitrate is formed there should be fixed carbon in these compounds. With pigeon breast muscle, however, only a small amount of carbon dioxide has been observed fixed (1), and apparently Reaction 1 does not occur. Fixation by oxalosuccinate carboxylase, Step 8, may occur nevertheless, for it would not be expected that there would be a large net uptake of carbon dioxide by this reaction, since the amount of citrate and isocitrate which accumulates in the oxidation is small. It is to be noted that the oxalosuccinate carboxylase reaction is an essential step in the tricarboxylic acid cycle itself, so even by this cycle carbon dioxide fixation is to be expected by pigeon breast muscle.

It is clear that the above comments concerning α-ketoglutarate synthesis are purely speculative. The discussion is intended only as an illustration of the development and basis of the cycle and to point out that in some respects it is tentative. It serves to emphasize the point of greatest experimental weakness in the Krebs cycle, i.e., the initial condensation. Thus far there is no *direct* proof that the tricarboxylic acids are formed by condensation of C_3 or C_2 and C_4 compounds and especially that this step is essential in oxidation. Such proof will require a study of the isolated reaction and elimination of the possibility of synthesis of isocitrate from α-ketoglutarate by carbon dioxide fixation.

The recent results of Weinhouse *et al.* (88) serve to emphasize the uncertainty regarding the initial condensation. These authors find, when carboxyl labeled acetate is oxidized by kidney slice in the presence of citrate, cis-aconitate, or α-ketoglutarate, that isotopic carbon is incorporated in the α-ketoglutarate but not in the citrate. This latter result was obtained when the citrate was added as such, or when aconitate was added and the citrate formed from aconitate was isolated. Weinhouse *et al.* (88) have concluded that citrate, isocitrate, and

aconitate are not in the direct path of acetate oxidation by kidney. They suggest the possibilities (a) that the tricarboxylic acid may be a phosphorylated derivative not in equilibrium with the parent substance, (b) that dehydrogenating coupling between oxalacetate and acetate might yield oxalosuccinate directly, (c) that acetate may couple with pyruvate directly to yield α-ketoglutarate without intervention of tricarboxylic acids.

It is clear that these results are a serious challenge to the occurrence of tricarboxylic acids as intermediates in the cycle. In the author's opinion the results would be more convincing if aconitate or isocitrate as such were isolated. It has been generally accepted that citrate is not an intermediate in the tricarboxylic acid cycle (10), (11), therefore it is questionable if this substance should be used as representative of the tricarboxylic acids. It is conceivable that the cis-aconitate, when added in high concentration, is rapidly converted to citrate and that subsequently the interconversions are not favorable for incorporation of C^{13}-acetate into the citrate.

The tricarboxylic acid cycle is the most complete explanation that has been given of biological oxidation up to the present date, and so far, with only minor modification, it has proved adaptable to all new developments. The scheme, therefore, should form a basis for future studies and can be modified to fit developments as they arise.

The remaining portion of this Section will be limited to a discussion of the cycle in relation to its application to specific problems and investigations.

The tricarboxylic acid cycle and oxidation of carbohydrate. In the scheme presented in figure 1 an attempt is made to give an integrated picture of the interrelationships of carbohydrate, fat and protein metabolism. The scheme, of course, is highly speculative and leaves many questions untouched. Nevertheless, a purpose is served in that it emphasizes an idea that is becoming increasingly apparent, that is, that fat, protein and carbohydrate metabolism have many points in common in the form of identical intermediate products (cf. Krebs (42)). Through these intermediates, interconversion of fats, carbohydrates and proteins may occur, and likewise through them oxidation may occur, in part at least, by essentially the same mechanisms. The "switch" from carbohydrate to fat oxidation, which is frequently spoken of, probably does not represent a major shift in mechanism but rather a change which is manifested through the same mechanism and may be produced by the differences in the degree of oxidation of the different substrates—carbohydrates as compared to fats. For example, carbohydrates and fats may both give rise to acetyl groups via Steps 4 and 18 and the acetyl group arising from either substrate may be oxidized by an identical mechanism. The fact that acetone bodies are formed from fat does not necessarily imply that there has been a switch in the fundamental mechanism of oxidation but rather it may imply that acetone bodies are formed from fat faster than they are broken down. This may occur because the rate of formation of acetyl groups from fat may be more rapid than is the oxidation via the cycle and the acetyl groups are therefore converted to acetone bodies. Likewise a respiratory quotient of 1.0

probably does not indicate that fat is not turning over and is not contributing to the oxidation product CO_2 but rather, it indicates that the net metabolism is equivalent to carbohydrate oxidation.

In considering the experimental basis for the cycle, some consideration already has been given to carbohydrate, i.e., pyruvate oxidation. For the present purpose it will be sufficient to consider the scheme only in relation to the isotope studies with labeled carbon dioxide. These investigations have, so far, been done almost entirely with pigeon liver; fixation by other animal organs, except heart (13), (19) has not been demonstrated. With pigeon liver acting on pyruvate, carbon dioxide is fixed in α-ketoglutarate (10), (11) and in the carboxyls of malate, fumarate, succinate and lactate (11).

In the cycle (fig. 1) starting with the conversion of pyruvate to oxalacetate by oxalacetate carboxylase (Step 1) the labeled carbon dioxide is fixed in the carboxyl adjacent to the methylene group of oxalacetate. By the reversible conversion of oxalacetate to fumarate, Steps 2 and 3, fixed carbon dioxide is distributed in both carboxyls of these C_4 dicarboxylic acids. If malonate is not present to inhibit the conversion of fumarate to succinate, the fixed carbon is likewise distributed in the succinate. The carboxyls marked "b" represent carboxyls which contain fixed carbon and if the rates of the reactions are adequate these carboxyls are in equilibrium with the carbon dioxide. The reversible conversion of oxalacetate to pyruvate and its reduction to lactate (Step 16) accounts for fixation in this compound. Likewise by the reversible reactions, the α and β carbons of the pyruvate lose their orientation in oxalacetate, and the carbons marked "a" theoretically represent the average of these two carbons. This latter point has not been checked as yet with an isotope tracer of these carbons.

Because there is some evidence in fatty acid oxidation that a C_2 compound enters the cycle (85), (88), (89) the primary condensation reaction, Step 5, is pictured as C_2 and C_4 addition to aconitate. The C_2 compound, which may be acetyl phosphate, is considered formed from pyruvate and fat by oxidation.

Krebs (92) has presented evidence that little exchange is to be expected between citrate and aconitate with pigeon liver because the rate of equilibration between citrate and cis-aconitate (Step 21) is slow as compared to the rapid rate of conversion of cis-aconitate to α-ketoglutarate. This observation is believed to account for the finding of substantially all the fixed carbon in only one carboxyl of the α-ketoglutarate (10), (11). If Step 21 was rapid all three carboxyls of the tricarboxylic acid would contain fixed carbon and the α-ketoglutarate formed by Step 8 would have fixed carbon in both carboxyls.

It is interesting that the discovery of fixation of carbon dioxide by the oxalosuccinate carboxylase reaction (Step 8) has not necessitated revision of any ideas on the cycle or invalidated any previous conclusions. It can be seen that Step 8 to the left inserts carbon dioxide into a carboxyl position, which already contains fixed carbon by virtue of the primary fixation (Step 1) and equilibria, Steps 2 and 3, and the additional, Steps 5, 6, and 7. The fixation by Step 8, thus, does not change the distribution of fixed carbon, except insofar as the rates may have been inadequate to equilibrate the carboxyls with carbon dioxide.

The oxidation of α-ketoglutarate to succinate, Step 10, eliminates the second "b" carboxyl and therefore the succinate formed from this compound contains no fixed carbon.

It is to be noted that the regenerated oxalacetate does not have fixed carbon introduced into its methylene and carboxyl groups. Therefore, repetition of the cycle cannot cause a new distribution of the fixed carbon in the components of the cycle. The cycle, thus, accounts for the known facts about the location of fixed carbon in components of the cycle.

No mention has been made in this discussion of the cycle about the occurrence of phosphorylated compounds. There is no definite information available on this subject although there is evidence that phosphorylation may occur. A phosphorylated compound may be formed in the oxalacetate β-carboxylase reaction, since ATP is a component of the system (25). The conversion of pyruvate, Step 4, may involve the formation of acetyl phosphate (33) but this reaction has not been verified with animal tissue. Furthermore, Ochoa (93) has found that in the oxidation of pyruvate to carbon dioxide by heart muscle extract, three atoms of phosphate are esterified per atom of oxygen utilized. The esterified phosphate was detected as ATP or hexosemonophosphate. This esterification is equivalent to fifteen atoms of phosphate per molecule of pyruvate. The same ratio of phosphate esterified to oxygen utilized has been observed by Ochoa (94) in the oxidation of the α-ketoglutarate to succinate. In the oxidation of succinate to fumarate the ratio is less and not more than one molecule of phosphate is esterified per atom of oxygen (95). The oxidation of malate to oxalacetate likewise causes phosphate uptake (96).

While there is little question that phosphate may be esterified in the oxidations of the cycle, there is little known about the mechanisms. Although part of the phosphate may enter into esterification with the components of the cycle during their dehydrogenation, it is not likely that more than a part of the phosphate is used for this direct phosphorylation of the substrate. No mechanism is known for such a large utilization of phosphate by this means. Part of the phosphate is probably used during the intermediate steps of transfer of the hydrogen to oxygen (93).

The mechanism of hydrogen transfer in the cycle has been studied by Krebs (97), (42). He has concluded that possibly all but one pair of the hydrogens pass through malate by the hydrogen carrier cycle of Szent-Györgyi (91). However, Ochoa (94) has now studied the one step concerning which Krebs was in doubt, i.e., the oxidation of α-ketoglutarate to succinate and has shown that the C_4 dicarboxylic acids are not involved in this oxidation. Therefore, it seems probable that only four pairs of hydrogen can be transferred by the malate-oxalacetate system in the tricarboxylic acid cycle.

In view of Ochoa's discovery of a new fixation reaction in the cycle it is interesting to consider whether fixation may occur in any of the other reactions. It will be noted that two Steps, 1 and 8, of the four decarboxylations have been shown to be reversible with animal tissue. On the other hand, Steps 4 and 10, the oxidation of pyruvate to a C_2 compound and of α-ketoglutarate to succinate have not.

There is some indirect though not conclusive evidence that these reactions are not reversible. In the experiments of Wood *et al.* (11) with pigeon liver, succinate was formed which contained excess C^{13} in the carboxyl groups. If Step 10 were reversible and α-ketoglutarate were formed from succinate, the excess C^{13} of the succinate would be incorporated in the α-ketoglutarate. This conversion apparently did not occur because there was excess C^{13} present in only the one carboxyl group, none being present in those carbons that would be derived from the succinate. Apparently if the reaction is reversible the rate was too slow to be detected under the conditions of this test.

The determination of the irreversibility of Step 4 is not direct and is not nearly as conclusive. In the experiments of Lorber *et al.* (76) in which $CH_3 \cdot C^{13}OOH$ was fed to rats the excess C^{13} was found exclusively in the 3 and 4 positions of the glucose unit of the liver glycogen. However, if Step 4 were reversible, it would be expected that pyruvate would result with excess C^{13} in the carbonyl group. If this compound were converted to glycogen, this excess C^{13} would be incorporated in the 1, 6 and 2, 5 positions of the glucose (cf. Section II, p. 218). Actually the dilution of the labeled carbon is so great in these experiments that the sensitivity of the test is low. There was a consistent slight excess of C^{13} in the 1, 6, 2, 5 positions of the glucose from these experiments, but it was less than the experimental error of the methods and was therefore not held to be significant. If Step 4 is reversible, it seems from these results that the rate must be quite slow as compared to the incorporation of the labeled carbon into the 3 and 4 positions. The concentration of C^{13} in these positions was definitely significant.

The tricarboxylic acid cycle and glycogen synthesis. The problem of glycogen synthesis and fixation of carbon dioxide has been considered in Section II, p. 216 and at this point the discussion can be limited to a consideration of the application of the findings to the tricarboxylic acid cycle.

With regard to the fixation of carbon dioxide in the 3 and 4 positions of glucose, it is evident that if Steps 1, 2 and 3 are reversible then pyruvate containing fixed carbon will be formed. It seems probable that part of this pyruvate will be converted directly to glycogen by reversal of glycolysis through Steps 15, 13, and 14. However, part of the pyruvate probably enters the glycogen after going through the tricarboxylic acid cycle. Since the dicarboxylic acids which are formed from α-ketoglutarate contain no fixed carbon, this mechanism would not provide any fixation of carbon dioxide in the glycogen.

Whether the C_4 dicarboxylic acids are in part converted to phosphopyruvate by some mechanism other than conversion to pyruvate and phosphorylation of the pyruvate with ATP (74) is not certain. Because Step 15 was believed to be irreversible (73), considerable speculation was aroused concerning alternate pathways for the formation of phosphopyruvate. The reaction which received the most attention for this transformation was the oxidation of fumarate. Kalckar (98) had demonstrated phosphopyruvate synthesis in fumarate oxidation and Lipmann (33) proposed a mechanism for this synthesis involving addition of inorganic phosphate to fumarate followed by oxidation to phospho-enoloxalacetate and then decarboxylation to phosphopyruvate. Carbon dioxide fixation

was proposed to play an essential rôle in this mechanism via Steps 1, 2, 3 and 12 (cf. Section II). However, Lardy and Ziegler's (74) findings of the reversibility of Step 15 have now removed the essential requirement for the fixation reaction. Moreover, the fact that Leloir and Munoz (99) have found that adenylic acid is necessary for formation of phosphopyruvate from succinate, fumarate, or citrate, indicates that a phosphate transfer, rather than a direct uptake of inorganic phosphate may occur in this formation of phosphopyruvate. In this case, the oxidation of the C_4 dicarboxylic acids may simply function to synthesize ATP for the phosphorylation of pyruvate or of C_4 acids. At present, the occurrence of phosphopyruvate formation by addition of inorganic phosphate *via* Step 12 must be considered speculative. It seems probable that carbon dioxide is fixed in glycogen by reversible equilibria 1, 2, 3, 15, 13, and 14, and that the obligatory function of carbon dioxide in glycogen synthesis is to be questioned.

Although the oxidative pathway of the tricarboxylic acid cycle does not have a direct part in the incorporation of fixed carbon into glycogen except as it may supply the energy for this synthesis, it seems likely to have a more direct function in the conversion of acetate to glycogen. Lorber *et al.* (76) (cf. Section II, p. 219) have shown that carboxyl labeled acetate fed to rats yields liver glycogen with labeled carbon in the 3 and 4 positions of the glucose unit. With acetate containing tagged carbon in both carbons of the acetate, the isotope was apparently in all positions of the glycogen (76).

If the acetate carbon is traced through the cycle of figure 1 it is clear that the observed distribution of labeled carbon results. The Steps are 19, 5, 6, 7, 8, 10, 11, 3, 2, 1, 15, 13, and 14. At the α-ketoglutarate step the carboxyl labeled acetate would give an α-ketoglutarate with tagged carbon in the carboxyl adjacent to the methylene group. This compound upon oxidation yields succinate with the labeled carbon in the carboxyl groups. Then in Step 1 carboxyl labeled pyruvate would be formed and this compound would yield glycogen with tagged carbon in the 3 and 4 positions of the glucose.

On the other hand, with the acetate with both carbons labeled, α-ketoglutarate would result with the carboxyl group and adjacent methylene group labeled. Succinate then would be formed, Step 10, with labeled carbon in the carboxyl and methylene groups. At Step 1, pyruvate would arise with tagged carbon in all positions, and from this pyruvate glucose would be formed with all positions labeled. The C^{13} was not found to be uniformly distributed in the glucose, however. In one experiment (76) for example, the following values were obtained for the excess C^{13} of the glucose: 3 and 4 positions 0.36, 2 and 5 positions 0.28 and 1 and 6 positions 0.22.

Explanation of this variation of the C^{13} is not difficult insofar as the 3 and 4 positions are concerned since the acetate carbon probably enters these positions by two mechanisms, i.e., by fixation of the $C^{13}O_2$ arising from the oxidation of the acetate and also by incorporation of the acetate carbon directly via the tricarboxylic acid cycle as outlined above. The combined effect of these two mechanisms could be to raise the C^{13} in the 3 and 4 positions above that of the other carbons of the glucose.

The explanation of the cause of the difference between the C^{13} content of the 2 and 5 positions and 1 and 6 positions is not nearly as evident. The α and β positions of the pyruvate formed by Step 1 of the scheme arise from the methylene carbons of the succinate. Since the succinate molecule is symmetrical, the C^{13} from the methylene carbons would of necessity be uniformly distributed in the α and β positions of the pyruvate and furthermore, via Steps 15 and 13, the distribution would be uniform in positions 1 and 6 and 2 and 5 of the glucose. Lorber, *et al.* (76) have therefore concluded that the tricarboxylic acid cycle as given in figure 1 may not be the sole route of conversion of acetate to glycogen. They point out, however, that the results can be accounted for by the tricarboxylic acid cycle if the scheme is modified. One modification that is suggested is of Steps 11, 3 and 2 of figure 1 to provide a pathway involving unsymmetrical molecules. This pathway may be in the form of phosphate esters of succinate and fumarate. However, it would seem necessary to assume that these unsymmetrical forms are in partial equilibrium with the symmetrical acids. This suggestion is indicated since the C^{13} concentration is not greatly different in the 1 and 6 and 2 and 5 positions, and therefore some randomization mechanism must be operating. Likewise in the scheme for carbon dioxide fixation in glycogen it has, thus far, been found necessary to assume that the oxalacetate is in at least partial equilibrium with a symmetrical molecule. This mechanism gives the necessary randomization of the fixed carbon in the carboxyl groups of oxalacetate so that pyruvate as formed from oxalacetate will contain fixed carbon.

It is obvious that the above explanation of acetate conversion to glycogen is entirely tentative. Of course, other schemes can be devised to fit the results. For example, α-ketoglutarate might be formed from pyruvate and acetate by Reaction 27. This α-ketoglutarate would be similar to that formed by the tricarboxylic acid cycle and thus would have the same limitations. For the present, however, since the tricarboxylic acid cycle is far more complete than other mechanisms, it seems advisable to use this cycle as a basis for speculation.

The conversion of carboxyl labeled butyrate and propionate to liver glycogen, likewise, was studied by Lorber *et al.* (76) and the labeled carbon was shown to occur in the 3 and 4 positions of the glucose unit. The mechanism of these transformations is not definitely known, but in the case of butyrate, conversion to acetoacetic acid and then to an acetyl group seems the most likely mechanism (cf. Fatty acid oxidation, p. 236). According to this proposal the acetyl group would enter the tricarboxylic acid cycle by the same reactions as carboxyl labeled acetate, and give rise to glucose with the tagged carbon in the 3 and 4 positions.

Blixenkrone-Möller (100) has suggested that the butyrate is converted to succinate by omega oxidation and that this is the reason butyrate is glycogenic. The change of succinate to glycogen most likely would be by oxidation to pyruvate through Steps 11, 3, 2, and 1, and this transformation, just as in the tricarboxylic acid cycle (via Step 15, etc.), would place the carboxyl carbon of the butyrate in the 3 and 4 positions of the glucose. However, preliminary experiments by Wood *et al.* (101) have indicated that this mechanism is not in agreement with their experimental observations. When they fed $CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot$

$C^{13}OOH$ to rats, the tagged carbon was found predominantly in the 3 and 4 positions whereas by the omega oxidation with succinate as an intermediate, the tagged carbon would be in all positions of the glucose. On the other hand, the results are in complete agreement with the predictions for conversion to two acetyl groups and transformation via the tricarboxylic acid cycle. The experiments are being repeated with butyrate containing a greater concentration of C^{13} than was available in the preliminary experiments. The sensitivity of the test will thus be greatly increased.

The mechanism for conversion of propionate to glycogen is generally considered to be via oxidation to pyruvate. The results of Lorber *et al.* (76) are in agreement with this concept.

Some discussion is perhaps desirable concerning the results obtained in isotope experiments in relation to past measurements of the glycogenic properties of compounds. The method used in the past has been to determine the *net* increase in glycogen after feeding a compound. However, it is now certain that the net increase in glycogen is not necessarily a measure of the amount of the fed compound which is incorporated in the glycogen. For example, Vennesland *et al.* (71) found when $C^{11}H_3 \cdot C^{11}HOH \cdot COOH$ was fed to rats that the liver glycogen deposited averaged 21 per cent of the fed lactate, but contained only 3.2 per cent of the lactate radioactivity. This fact emphasizes that when a compound is fed and an increase is found in a body component, it does not follow that the carbon of the fed compound is transferred as such to the increased component.

Schoenheimer and Rittenberg (102) have presented evidence that the components of the body are in dynamic equilibrium and are constantly "turning over". Therefore, when a compound is fed it enters this metabolic "pool" and is converted into many different components of the body. If the fed compound enters into the dynamic equilibria of those compounds which are precursors of glycogen, it is to be expected that the compound will become mixed with the other precursors in the metabolic pool. Vennesland *et al.* (71) believe this dilution by mixing with precursors is the explanation for the small incorporation of C^{11} from the C^{11}-lactate, as compared to the much greater observed net increase in the liver glycogen.

Actually, the feeding of a compound could influence a body component in any one of the following ways:

a. The compound fed could be transformed into the body component and a *net* increase in the component occur.

b. The compound fed could be transformed into the body component but *no net* increase in the component occur.

c. The compound fed could cause a net increase or decrease in the body component without itself entering the body component.

In the latter case the fed compound would not enter the metabolic pool of precursors which serve as a source of carbon for formation of the body component, but rather by indirect action would influence the steady state of the dynamic equilibria involved in formation of the body component. Presumably such a case could arise when the fed compound produced high energy phos-

phate such as ATP which was used in turn in synthesis of the body component. Viewed in the light of these considerations the explanation of Buchanan *et al.* (85), of why fed acetate does not usually lead to a net increase in glycogen, may be an over simplification. They propose that acetate is oxidized in the tricarboxylic acid cycle to two molecules of carbon dioxide and that one molecule of oxalacetate is regenerated. Since there is no net increase in the oxalacetate, which is considered to be the carbohydrate precursor, no net increase in glycogen is considered likely to occur. This view overlooks the fact that the dynamic equilibrium may be subject to change not only because of the concentration of the actual carbon precursors, but also, for example, because of the energetics of the system, ATP concentration, etc.

It is clear from the above discussion that the amount of isotope incorporated into glycogen likewise is not a direct measure of the net increase in glycogen. This erroneous assumption has been made by Deuel *et al.* (103) in interpreting the experiments of Buchanan *et al.* (72) on butyrate conversion to glycogen. Furthermore, the finding of Lorber *et al.* (76) that C^{13}-acetate is converted to glycogen does not conflict with the results of feeding experiments which in general, have been considered to indicate that acetate is non-glycogenic (104), (105). It is only necessary to assume that when acetate is fed and enters glycogen it does not shift the dynamic equilibria in such a way as to cause a *net* increase in glycogen.

The tricarboxylic acid cycle and oxidation of fatty acids. The oxidation of fatty acids via the tricarboxylic acid cycle has only recently been given serious consideration. This particular phase of fat oxidation will be considered in some detail in this review but for a more complete account of the general subject of fatty acid metabolism see Stadie's recent review (106).

The evidence of fatty acid oxidation by the tricarboxylic acid cycle has been the result of accomplishments in a number of different fields, and much additional work is necessary before uniform agreement can be reached on the mechanism of the reactions. The greatest divergence of opinion concerns the type of compound which is formed in the original condensation to give a tricarboxylic acid.

Consideration of the subject of fatty acid oxidation by the tricarboxylic acid cycle will take the following order:

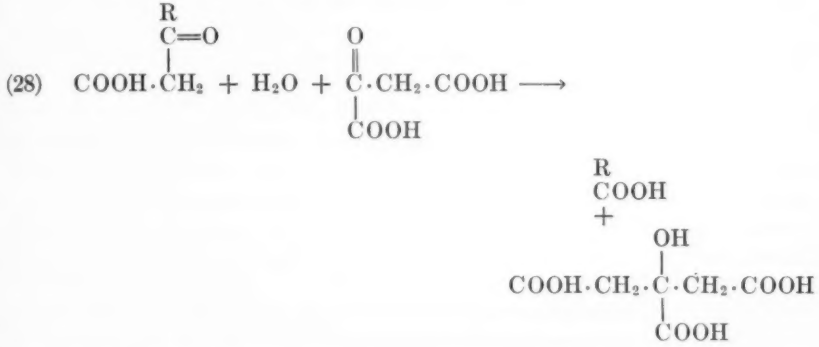
a. The initial condensation reaction will be discussed; particularly the evidence will be considered in relation to a possible condensation of C_2 and C_4 compounds.

b. Experiments will be cited which show that C_2 compounds are rapidly formed from fatty acids, therefore the oxidation of fatty acid via the tricarboxylic acid cycle would not be limited by this reaction.

c. The evidence will be presented which shows that acetate and acetoacetate enter into formation of the intermediary compounds of the cycle, thus providing evidence that the C_2 component as formed from fat may be oxidized by the cycle.

a. *The initial condensation reaction.* The three mechanisms for the primary condensation proposed by Breusch (83), Lynen (87), and Wieland and Rosenthal (84) have received the most attention.

Breusch (83) suggested that the fatty acids, after conversion to β-keto acids, condense with oxalacetate according to Reaction 28.



Breusch called the enzyme citrogenase. Citrogenase is presumed specific for β-keto acids but not for the R group. For example, several straight and branched chained monocarboxylic, as well as dicarboxylic β-keto acids are stated to yield citrate with the enzyme. Furthermore, the R-COOH acid resulting from Reaction 28 after undergoing a second β-oxidation is suitable for the additional oxidation by Reaction 28. Thus complete oxidation of the fatty acid can be accounted for by the scheme.

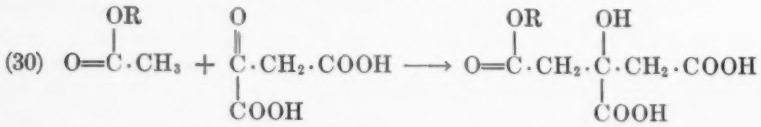
The enzyme, citrogenase, was extracted with 0.5 per cent $NaHCO_3$ and was found in large amounts in muscle, kidney and brain, but there was little in liver and none in spleen, pancreas and lung. Unfortunately only the single report which contained no experimental data has thus far been available for evaluation of this work.

Breusch apparently does not believe that the tricarboxylic acid cycle is important in carbohydrate oxidation. The basis of his criticism is not clear to the author. It is very difficult to understand how he can justify the scheme for fat oxidation and in turn criticize it as an explanation for carbohydrate oxidation. The fact that evidence has been obtained that citric acid is not a component of the mechanism of pyruvate oxidation is not a logical reason to discard the entire scheme for carbohydrates.

The second mechanism of condensation was proposed by Lynen. Lynen studied the oxidation of acetate by yeast and has presented evidence that the oxidation occurs via the tricarboxylic acid cycle (107), (87). The inhibition of yeast oxidation by malonate (87) was demonstrated for the first time. This was accomplished by lowering the pH to about 2; at this pH the yeast cell is permeable to malonate.

Lynen suggested that the oxalacetate and acetate condensation is coupled with the dehydrogenation of aldehydes (107). An R group (perhaps a phosphate) is suggested to be linked either to the carboxyl of acetate or of oxalacetate. This combination with R is believed to activate the acid so that condensation

can occur. On the assumption for purposes of illustration that the acetate is the molecule that is activated, then the reaction would be as follows:



It is suggested that the procitrate, thus formed, is converted to a proaconitate and then by splitting off the R, aconitate is formed. The remainder of the scheme is by the usual conversions of the tricarboxylic acid cycle.

The advantage claimed for this scheme is that it provides an unsymmetrical citrate molecule which makes possible the explanation of earlier results obtained by Sonderhoff and Thomas (108), with $CD_3 \cdot COOH$. The results of Sonderhoff and Thomas have been discussed previously on p. 212. Likewise the scheme can be used to explain the occurrence of fixed carbon dioxide in only one carboxyl of α-ketoglutarate as found in the pigeon liver experiments (10), (11). This is because the scheme avoids the randomization of the fixed carbon which would occur if the symmetrical citrate was an intermediate.

Wieland and Rosenthal (84) have proposed a third mechanism for the condensation reaction leading to formation of tricarboxylic acids. Wieland and Gottfried (109) had found that acetate is rapidly oxidized by kidney cortex; and since it was known that acetate could be converted by some tissues to acetoacetate, the oxidation of this latter compound was studied next (84). With kidney slices and a combined substrate of oxalacetate and acetoacetate, oxygen uptake was increased 40 per cent above the endogenous rate, whereas either substrate by itself caused only a 10 per cent increase. When barium ions were added to inhibit citrate breakdown, considerable citrate was found formed.

On the above basis Wieland and Rosenthal postulate that oxalacetate and acetoacetate can combine to form either citroylacetate or acetyl citrate. These compounds on hydrolysis would yield acetate and citrate. Since acetate was not found as an end product it was suggested that the product of the first condensation with oxalacetate might combine with a second molecule of oxalacetate to give a double condensation compound which on hydrolysis would yield two molecules of citrate and no acetate.

The yield of citrate with oxalacetate and pyruvate was about 50 per cent as large as with acetoacetate. It was suggested that pyruvate might first be converted to acetoacetate and then form citrate.

By conversion of the unsymmetrical addition products to aconitate, the fixation experiments and deuteroacetate results of Sonderhoff and Thomas could be explained just as in the case of Lynen's scheme.

The enzyme catalyzing this reaction was found in kidney and heart but not in liver.

It is obvious on critical examination of the above results that the proposed mechanisms for citrate formation are purely speculative. Actually, all that is known is that citric acid is formed from oxalacetate when in combination with a

number of other compounds such as *acetate, pyruvate, acetoacetate and other β-keto acids.*

In practically every case oxygen was found to facilitate the reaction, whereas no function has been indicated for any oxidation in the reaction.

The fact that liver does not form citrate from acetoacetate (83), (84) has been considered an indication that liver lacks citrogenase, whereas it is just as probable that the liver preparations only lack enzymes for converting acetoacetate to an intermediate capable of taking part in the citrogenase reaction. At any rate, there is an enzyme in liver which forms citrate from pyruvate and oxalacetate and there is no reason to believe it is different than the enzyme which produces citrate in kidney and heart. In fact, until evidence is presented to the contrary, it is probably a fair assumption that the same enzyme causes condensation in each case.

The results of Hunter and Leloir (86) are of considerable interest in this connection. They have recently demonstrated with a washed suspension of kidney cortex that α-ketoglutarate oxidation stimulates the conversion of acetoacetate and oxalacetate to citrate. This function of α-ketoglutarate is reminiscent of Lynen's (107) observation of the activating effect of oxidation of succinaldehyde on citrate formation by yeast. On the other hand, Hunter and Leloir found that α-ketoglutarate oxidation was not necessary for the formation of citrate when oxalacetate was the only substrate. Oxalacetate breaks down to pyruvate spontaneously and the reaction in the latter case may have been by union of oxalacetate and pyruvate or a product formed from pyruvate. The important point is that acetoacetate apparently undergoes some primary reaction facilitated by α-ketoglutarate oxidation before it can be converted to citrate. It therefore seems quite unlikely that acetoacetate is a *direct* intermediate in the citrogenase reaction.

Additional evidence that acetoacetate is not an intermediate has likewise been presented by Lehninger (110). Using a liver preparation he found that citrate was formed oxidatively from either pyruvate or octanoate, when added in combination with fumarate. On the other hand, when fumarate was omitted, citrate was not formed, but there was increased formation of acetoacetate as compared to that formed when the fumarate was added. From acetoacetate and fumarate the formation of citrate was no greater than from fumarate alone. These results are interpreted as evidence that acetoacetate is not a component of the citrate reaction but is formed when oxalacetate is not present to shift the reaction toward formation of citrate. It is suggested that pyruvate and fatty acids may have a common C_2 intermediate which react in the condensation.

In connection with pyruvate conversion to citrate, it is to be noted that Martius (111) has presented evidence that the 7 carbon compound, 2-keto-4-hydroxyadipic acid is not precursor of citrate. This compound was not converted to citrate by kidney preparation. It is of interest that Bloch and Rittenberg (112) have presented evidence that pyruvate as such may react with amines in acetylation and that the pyruvate is broken down to an acetyl group after combination with the amine. It is conceivable that the same type of reaction

may occur in the condensation of pyruvate to form tricarboxylic acids; i.e., the pyruvate may react prior to formation of the C_2 fraction.

Hunter and Leloir (86) and Lehninger (110) have tested acetyl phosphate as a probable C_2 component for the citrogenase reaction and have found it inactive. However, since acetyl phosphate is rapidly broken down by animal tissue (41), it is questionable whether the test will have any definite significance, until it is demonstrated that the ester was sufficiently stable under the conditions of the test and available for the enzyme reaction.

It is evident that information concerning the initial condensation reaction is in a very confused state at present. It is obvious that considered as a group, the compounds (acetate, pyruvate, acetoacetate) which form citrate in combination with oxalacetate have one thing in common, i.e., they are all potential sources of C_2 compounds. Further investigation is needed, however, before any exact idea can be reached as to the mechanism of the reaction.

b. *Evidence of formation of C_2 compounds in the oxidation of fatty acids.* An interesting feature of biochemistry, that has become apparent from recent isotope studies, is the remarkable ability of animals to construct complex compounds from small units, such as C_2 and C_1 compounds. There is increasing evidence that the food is dissimilated and then synthesized to body constituents. Of course there are many exceptions; the essential vitamins and amino acids must be added as more complicated molecules. Even here it is often true that specific groups are essential components rather than the whole molecule. It is very significant that acetic acid is incorporated into such compounds as cholesterol (113), (112), hemin (112), fatty acids (114), glycogen (76), glutamate and aspartate (89) and that glycine is converted to glycogen (115), porphyrin (116) and creatine (117). Furthermore, Bloch and Rittenberg (112) have recently calculated that from 0.9 to 1.2 grams of acetate may be formed daily per 100 grams of rat tissue (cf. p. 239 for further discussion). Thus the C_2 compound clearly plays an important part in animal metabolism.

The evidence that C_2 compounds are formed from fatty acids dates back to Knoop's studies of β-oxidation (118). Schoenheimer and his collaborators have extended these observations with the more nearly natural compounds, the deuterio labeled acids, and have demonstrated that the long chain acids are built up and broken down by units of 2 carbons. For example, deuterio-palmitic acid, 16 carbons, was converted to deuterio-stearic acid, 18 carbons, and deuterio-stearic acid was converted to deuterio-palmitic acid (119, 120).

Very recently Rittenberg and Bloch (114) have presented convincing evidence that acetate is converted to fatty acids. Sodium acetate containing 77 per cent deuterium in the methyl group and 19.6 per cent C^{13} in the carboxyl group was fed to mice in a high carbohydrate, low fat diet at the rate of 1.6 mM of acetate per 100 g. of weight per day for eight days. The respiratory carbon dioxide at the conclusion of the experiment contained 0.066 per cent excess C^{13} and the deuterium concentration in the body fluids was 0.09 per cent excess. The fatty acids were isolated from the liver and carcass. The total fatty acids of the carcass contained 0.081 per cent C^{13} and 0.13 per cent deuterium while the fatty

acids of the liver contained 0.103 per cent C^{13} and 0.32 per cent D. These results show that both the deuterium and C^{13} labeled carbon atoms of the acetate were used in the synthesis of fatty acids. Since the respiratory carbon dioxide contained less C^{13} than the fatty acids it was concluded that not all of the carbon of the acetate was incorporated as carbon dioxide.[5]

The saturated fatty acids were isolated and then decarboxylated. The concentration of C^{13} in the carboxyl carbon of the saturated acids was found to be almost twice as high as the average C^{13} of the entire molecule. The most plausible distribution which would explain these results is one in which the isotopic carbon is in every other carbon atom, i.e., the odd carbon atoms of the fatty acid. This proposal is supported further by the fact that when the unsaturated "oleic acid" fraction was oxidatively split into "pelargonic" and azelaic acid fractions and analyzed, both the C^{13} concentrations and deuterium concentrations were nearly the same in the two fractions. Thus the isotopes were distributed in the whole carbon chain. This fact indicates the complete molecule was formed with incorporation of acetate carbon. Rittenberg and Bloch have estimated that at least one-fourth of all the carbon atoms of the synthesized fatty acids were derived from acetate carbon.

These findings can be taken to indicate that the reactions of fatty acid degradation to C_2 compounds are reversible, and that fats may be synthesized by these reversible reactions. The condensation of acetate to acetoacetate may be the first step in the synthesis. The fact that Swendseid *et al.* (121) have demonstrated *in vivo* that acetate is converted to acetone bodies gives support to this idea.

It is interesting that Wood *et al.* (122) obtained convincing evidence with $CH_3C^{13}OOH$ that the butyl alcohol bacteria convert acetate to butyric acid and butyl alcohol. The reaction is similar to the above condensation since the C^{13} was equally distributed in the carboxyl and beta positions. Also the results of Barker, Kamen and Bornstein (137) are significant in this connection. Using *Cl. kluyveri*, which ferments alcohol and acetate to butyrate and then to caproate,

$$(31) \qquad CH_3 \cdot CH_2OH + CH_3 \cdot COOH \rightarrow CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH + H_2O$$

$$(32) \qquad CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH + CH_3 \cdot CH_2OH \rightarrow CH_3 \cdot (CH_2)_4 \cdot COOH + H_2O$$

[5] Although the above conclusion is very likely correct it should be pointed out that based on this single observation there would be a possibility of error. It is conceivable, that the rate of exchange at the site of oxidation of acetate and synthesis of fat may not be rapid enough to bring the components involved into complete equilibrium with those of the body in general. In other words, the concentration of C^{13} and D in the bicarbonate and water respectively of the organ or site where the oxidation is occurring might be higher than that of the respiratory carbon dioxide and average body water. In this case an isotopic compound could be synthesized by incorporation of $C^{13}O_2$ and HDO and have a higher content of C^{13} and D than that of the respiratory carbon dioxide and body water. The possibilities of such an error are emphasized by Kalckar *et al.* (140) and Furchgott and Shorr (141), who showed that the isotopic concentration in the intracellular and extracellular phosphate could be quite different, and that this difference probably accounts for the observations by Sacks (142) which seemed to indicate that the Meyerhoff-Warburg scheme of glycolysis did not apply to the working muscle.

it was shown that in the presence of acetate labeled with C^{14} in the carboxyl group, the C^{14} was approximately equally distributed between the carboxyl and beta carbons of the butyrate. In the caproate one-third of the C^{14} was in the carboxyl group, the rest was presumably in the beta and delta positions. As the fermentation proceeded the concentration of C^{14} in the acetate dropped, which indicated there was oxidation of the ethanol to acetate or a compound in equilibrium with the acetate. When the organism was grown in normal ethanol and carboxyl labeled butyrate, isotopic caproate was formed but not acetate. The C^{14} in the caproate was not in the carboxyl but presumably was in the beta position. The condensation therefore involves butyrate or a related C_4 compound and the methyl group of acetate or a reactive derivative formed by oxidation of alcohol. This bacterium thus produces a reaction which is very similar to the conversion which occurs in fatty acid synthesis by animals.

Closely related to the above considerations is the mechanism of acetoacetate formation from fatty acids. The original β-oxidation theory assumed that there was successive splitting off of C_2 units in the form of acetate until four carbons remained, and that this 4-carbon chain was then oxidized to ketone bodies. It became obvious that this theory was not adequate, when Jowett and Quastel (123) found that more than one ketone body was formed per molecule of fatty acid dissimilated. The idea of a 4-carbon cleavage of the molecule was therefore suggested.

MacKay *et al.* (124) proposed, however, that the conversion was to acetate and that the acetate was then converted to acetone bodies. Weinhouse, Medes and Floyd (125) have now presented convincing evidence of this mechanism of formation of acetone bodies from fatty acids. When n-octanoate labeled by the incorporation of C^{13} in the carboxyl group was incubated *in vitro* with liver slices the resulting acetoacetate contained the excess C^{13} equally distributed between the carbonyl and carboxyl carbon atoms. The only mechanism which satisfactorily explains these results is one which involves random coupling of C_2 units as illustrated in Reaction 33.

(33) $$CH_3 \cdot (CH_2)_6 \cdot C^{13}OOH \rightarrow 4CH_3 \cdot C^{13}OOH \rightarrow 2CH_3 \cdot C^{13}O \cdot CH_2 \cdot C^{13}OOH$$

The C_2 unit may not be acetic acid, but it is a likely possibility in view of the results of Swendseid *et al.* (121) and Weinhouse *et al.* (126) showing the C^{13}-acetate is converted to C^{12}-acetone bodies.

It does not necessarily follow that none of the acetoacetic acid can be formed by direct conversion from a C_4 intermediate compound. In fact, Medes *et al.* (127) have demonstrated with carboxyl labeled butyrate that about 22 per cent was converted by direct oxidation to acetoacetate by the liver slices. The acetoacetate formed by this conversion contains C^{13} in the carboxyl group only, and for that reason the resulting acetoacetate contained more C^{13} in the carboxyl group than in the carbonyl group. The reaction may be as follows:

(34) $$CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH \rightarrow CH_3 \cdot CO \cdot CH_2 \cdot COOH \leftrightharpoons CH_3 \cdot COOH$$

In the case of octanoate, since the last 4 carbons did not contain any labeled carbon, the direct conversion could not be detected.

These results, beyond their direct importance in contributing information concerning acetone body formation, give good evidence that C_2 components are formed from fatty acids.

Bloch and Rittenberg (128) have made an important contribution to information on the formation of C_2 compounds from different compounds by using intact rats. This was done by studying the rate of acetylation of phenylaminobutyric acid, when this compound and the test compound were included in the diet. The compounds were labeled with deuterium and the amount of deuterium excreted as acetylphenylaminobutyric acid was determined. The findings are interpreted on the assumption that acetic acid, or some closely related compound, is the direct acetylating agent and that the appearance of deuterio acetyl groups is proof of the intermediary formation of deuterio acetic acid from the isotopic test substance.

Deuterio acetyl groups were excreted after administration of labeled ethanol, butyrate, alanine, n-valerate and myristate, but not after propionate and 10, 11-dideuterio undecylate. The results are considered to be in complete agreement with the theory of β oxidation to C_2 units. The negative results with 10, 11-dideuterio undecylic acid are believed to result because the 9, 10 and 11 carbon atoms are converted to propionate. This compound did not yield an acetyl group. The fact that propionate did not form an acetyl group is considered evidence that propionate is not oxidized to pyruvate (Step 23 of fig. 1). If pyruvate was formed from propionate it is quite certain that the foreign amine would have been acetylated by the resulting pyruvate. It is assumed that pyruvate, if formed, would acetylate since alanine is an effective acetylating agent and is believed to acetylate after its conversion to pyruvate. Bloch and Rittenberg suggested therefore that the oxidation of propionate must be at the β carbon and not at the α carbon in the conversion of propionate to glycogen. It is interesting in relation to this point to note (Section II, p. 214) that in the propionic acid fermentation the reverse reaction, conversion of pyruvate to propionate is not believed to occur by a direct reduction.

Bloch and Rittenberg (112) have extended these studies of the acetylation of foreign amines by use of acetic acid labeled with deuterium in the methyl group and C^{13} in the carboxyl group. They have shown that there is no substantial change in the ratio of deuterium and C^{13} during acetylation in the animal body. This fact indicates that there is no loss of deuterium by exchange during the acetylation reaction. It was necessary to establish this fact, since these authors were attempting to determine the amount of acetate formed in the body by measurement of the dilution of the deuterium in the acetylated amine as compared to that of the fed acetate. The basis of the calculation is that the dilution is caused entirely by mixing of the administered acetate and acetate occurring in the animal body. The above results showed that the dilution could not have been by loss of deuterium from acetate through exchange reactions. After excluding this possibility it was necessary to assume that acetate is the only acetylating agent and that other compounds do not react with the amine and then become converted to an acetyl group. Bloch and Rittenberg believe this

is true for aromatic amines, although they showed alanine will apparently acetylate phenylaminobutyric acid in a form other than acetate. Their calculations indicate that from 15 to 20 mM of acetyl groups are formed per 100 grams of rat tissue per day.

There are a number of possibilities for error in this determination. Obviously any mechanism that removes the administered acetate without its coming to equilibrium with the acetyl groups will cause error. This would be the result if acetylation involved formation of an intermediate and if acetate was oxidized or removed without coming to equilibrium with this intermediate. The dilution by body acetyl groups would, in this circumstance, appear greater than is actually the case. The opposite kind of error would be made if the C_2 compound, as formed in the cell, was oxidized at the site of formation without equilibrating with added acetate or with the C_2 compounds at the site of acetylation. In this case the calculated value of acetyl groups would be too low. It should be borne in mind that acetate as such may be a dead end or side reaction, and that acetate may be converted to an intermediary C_2 component of the body but as such may not be the true intermediate of oxidation. Nevertheless, the experiments of Rittenberg and Bloch are the first attempt to measure formation of C_2 compounds in the body and the results indicate there is a substantial amount of C_2 compounds formed in animal metabolism.

It is interesting that Bloch and Rittenberg's results exclude the conversion of acetate to acetoacetate in the acetylation reaction. If this reaction occurred there would be a loss of deuterium and, therefore, a change in the ratio of C^{13} to deuterium in the acetylated amine.

They suggest that the major part of the acetyl group arises by oxidation of fatty acids. The mechanism of the reactions by which the fatty acids are oxidatively degraded to C_2 units has not been investigated extensively. No intermediate products of β oxidation have been isolated; likewise, it has not been possible to detect the lower fatty acids in oxidations by liver preparations (125), (129).

Lehninger (130) has demonstrated that adenosine triphosphate is required in oxidations by liver homogenates. He suggests that an acyl phosphate is formed and that this phosphorylation activates the molecule for oxidation.

c. *Formation of intermediate compounds of the cycle from labeled acetate and acetoacetate.* Aside from the evidence that fatty acids may be converted to C_2 compounds, there is additional evidence that fatty acids may be dissimilated by the tricarboxylic acid cycle, in that some of the intermediate steps of the cycle have been demonstrated. The most convincing evidence has been supplied by Buchanan *et al.* (85) using labeled acetoacetate and acetate and by Rittenberg and Bloch (89) and Weinhouse *et al.* (88) using labeled acetate. Prior to these experiments, there was ample evidence that the intermediate compounds of the cycle were rapidly fermented (79), (80), (42), (131), (107) by different tissues. Only the evidence with labeled compounds can be considered in detail in this report.

Buchanan *et al.* (85) found that citrate, α-ketoglutarate, succinate, fumarate, malate or oxalacetate stimulated the oxidation of acetoacate by homogenized guinea-pig kidney cortex. As a test of whether or not acetoacetate was oxidized by the tricarboxylic acid cycle the amount of conversion of $CH_3 \cdot C^{13}O \cdot CH_2 \cdot C^{13}OOH$ to intermediates of the cycle was measured. The isotopic acetoacetate was oxidized in mixture with succinate in one case and α-ketoglutarate in another. Fumarate was isolated from the experiment to which succinate was added and α-ketoglutarate from the other experiment. The isolated acids contained an excess of C^{13} of such a magnitude as to indicate that the acetoacetate was oxidized *via* the tricarboxylic acid cycle.

By degradation of the isolated acids the position of the C^{13} was determined. The excess C^{13} was in the carboxyl groups of the fumarate. In the α-ketoglutarate the carboxyl group adjacent to the keto group contained 0.24 per cent excess C^{13} and the remainder of the molecule 0.59 per cent. If all of the excess C^{13} was in the two carboxyl groups of the α-ketoglutarate, the excess C^{13} in the second carboxyl group would be 2.36 per cent.

When carboxyl labeled acetate was oxidized together with α-ketoglutarate, succinate was formed which contained excess C^{13}. In a similar experiment by Weinhouse *et al.* (88) with labeled acetate and rat kidney mince, the α-ketoglutarate was isolated and degraded. One-sixth of the total C^{13} in the α-ketoglutarate was found in the carboxyl adjacent to the carbonyl group, the remainder is presumed to be in the other carboxyl group.

Weinhouse *et al.* (88) by a similar technique were unable to show the conversion of acetate to citrate when either citrate or aconitate was added together with C^{13}-acetate. The significance of these results has been considered on p. 224.

The above results with α-ketoglutarate and the dicarboxylic acids are in complete agreement with the proposals of the scheme of figure 1. The α-ketoglutarate and succinate would, according to this scheme, be oxidized to oxalacetate by Steps 10, 11, 3, and 2. The resulting oxalacetate would unite with an acetyl group containing C^{13} in the carboxyl group; the latter being formed from the acetoacetate by Step 18. Then by Steps 5, 6, 7, 8, 10, 11, α-ketoglutarate and fumarate would be formed. The α-ketoglutarate would contain excess C^{13} only in the carboxyl group distal to the carbonyl group and the fumarate would contain isotope only in the carboxyl groups. If this C^{13}-fumarate re-entered the cycle and reacted with a C^{13}-acetyl group the resulting α-ketoglutarate would contain excess C^{13} in both carboxyl groups. This may in part account for the occurrence of some isotope in the carboxyl adjacent to the keto groups in α-ketoglutarate. Partly the distribution in both carboxyls may result from the reversible side reaction to citrate, Step 21.

The results obtained by Rittenberg and Bloch (89) are very similar and were obtained by feeding acetate labeled with C^{13} in the carboxyl group to rats and mice. The intermediates of the tricarboxylic acid cycle could not be isolated from the intact animal but glutamic and aspartic acids, which are in biological

equilibrium with α-ketoglutarate (Step 9, fig. 1) and oxalacetate respectively, were isolated. The carboxyl adjacent to the amino group in the glutamate, which was isolated from mouse liver, contained 0.096 per cent excess C^{13} and the entire molecule contained 0.080 per cent. If the excess C^{13} was entirely in the two carboxyl groups, the carboxyl distal to the amino group would have contained 0.304 per cent excess C^{13}. The aspartate contained 0.038 per cent excess C^{13}. This compound was not degraded.

It should be mentioned that the C^{13} in the glutamate was greater than that in the respiratory carbon dioxide. Therefore, the C^{13} could not have been incorporated in the glutamate exclusively as carbon dioxide. Presumably, however, some of the C^{13} in the α carboxyl of the glutamate may have been incorporated by carbon dioxide fixation.

It is evident that these results are almost identical with those obtained by Buchanan *et al.* (85) and Weinhouse *et al.* (88). The low concentration of C^{13} found in the isolated compounds is to be expected because of the large dilution of acetate in the animal body and because the C^{13}-glutamate and asparate would be diluted by normal glutamate and asparate of the tissue. The data are particularly important because they show that results obtained with the intact animal are adaptable to the tricarboxylic acid cycle.

These results do not confirm Krebs and Eggleston's (132) and Weil-Malherbe's (133) contention that acetoacetate is not oxidized by the citrate cycle. Krebs suggested that the increased production of citrate from oxalacetate and acetoacetate as compared to that from oxalacetate alone, was caused simply by the acetoacetate acting as a hydrogen acceptor and that the acetoacetate was not oxidized by the cycle.

SUMMARY. The experimental evidence for the tricarboxylic acid cycle has been critically examined and the point of greatest weakness in the evidence is considered to be that concerning the initial condensation reaction to form a tricarboxylic acid.

The adaptability of the tricarboxylic acid cycle as an explanation of carbohydrate and fatty acid oxidation is discussed particularly in relation to recent observations made by isotope experiments. The scheme satisfactorily accounts for the position of carbon dioxide fixed in pyruvate oxidation by pigeon liver. Likewise, the formation of glycogen from acetate, propionate and butyrate can be explained by this mechanism, although for this explanation a slight modification of the scheme seems necessary.

Oxidation of fatty acids via the tricarboxylic acid cycle is a likely possibility. By assuming that the initial condensation reaction of the cycle involves a C_2 compound, an integrated picture of carbohydrate and fatty acid oxidation can be obtained. The proposal that the initial condensation is a more complicated process than a C_2 and C_4 condensation is considered inadequately supported by experimental evidence. For the sake of uniformity and simplicity, it is deemed advisable at present to picture the condensation to be with a C_2 component. Fatty acid oxidation via the cycle can be explained since C_2 components are rapidly formed from fatty acids and also intermediate compounds of the cycle

are formed with incorporation of the carbon from the C_2 components in the predicted positions.

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THE EFFECT OF BODY TEMPERATURE ON DRUG ACTION

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The action of drugs in animals is influenced by body temperature as well as by the more commonly recognized variables such as body weight, sex and species. In poikilotherms, the temperature of the body is dependent upon that of the environment and may be varied over a range of about 35°C. In homeotherms, the fluctations in body temperature are so slight that the influence of temperature upon pharmacological action is difficult to measure in completely normal animals, and is usually neglected. It is only when the body temperature deviates markedly from normal that the effect of such changes becomes important. It has only recently been appreciated that human beings can recover from body temperatures as low as 23°C. (Talbott, 1941), although this was known much earlier (Reincke, 1875). Body temperature actually can be varied over a range of about 20°C. in man and about 35°C. in some other non-hibernating mammals. Since such ranges of temperature are possible, the influence of body temperature upon drug action becomes of considerable importance in homeotherms as well as in poikilotherms.

The effect of temperature upon drug action has been investigated under three distinctly different circumstances: (a) in homeotherms at varied levels of environmental temperature, (b) in both homeotherms and poikilotherms at varied body temperatures, and (c) in isolated organs and tissues at graded temperatue levels. This review includes chiefly the alterations in drug action in vertebrates resulting from changes in body temperature. Results obtained at varied environmental temperatues and on isolated preparations are included in some cases to supplement these observations.

One approach to the problem of the effect of temperatue upon drug action is the determination of the extent to which temperature coefficients may be modified by the presence of drugs. Some examples of such results, obtained chiefly on invertebrates, are cited by Bělehrádek (1935) and Crozier (1924). The effect of the following substances on the temperature coefficient of the heart beat of the frog has been reported: epinephrine, tyramine, ethyl alcohol, guanidine, atropine, caffeine, pilocarpine, camphor and inorganic ions (cf. Bělehrádek, 1932). For methods of calculation and interpretation of temperature coefficients the reviews by Przibaum (1923), Kanitz (1915; 1925), Bělehrádek (1935), Hoagland (1936), Sizer (1943) and van Rysselberghe (1944) may be consulted. Of particular interest are the recent studies of temperature and the action of narcotics and other drugs on bacterial luminescence (F. H. Johnson et al., 1942; 1943; McElroy, 1943), and the application to these data of the theory of absolute reaction rates (cf. Glasstone et al., 1941; Eyring and Magee, 1942).

It has not been possible to include all papers in the literature dealing with temperature and drug action. In particular, many of the older references cited

by Brunton (1885), Richet (1889), Saint-Hilaire (1893), Langlois and Richet (1895) and Matisse (1919) have been omitted. The effects of environmental temperature on vitamin requirements and the effect of temperature on bactericidal action are not included.

PHYSIOLOGICAL EFFECTS OF TEMPERATURE. The effects of temperature on drug action, particularly in homeotherms, cannot properly be evaluated without first considering the effect of changes in body temperature upon physiological processes. The alteration in pharmacodynamic response resulting from lowering of body temperature, for example, is not due entirely to the simple effect of temperature upon the rate of chemical reactions, but is often greatly modified by circulatory, nervous or metabolic changes resulting from the decrease in temperature. For this reason, certain temperature effects, uncomplicated by the action of drugs, and ranges of body temperature compatible with vertebrate life, are discussed below.

Poikilotherms. Under laboratory conditions the body temperature of poikilotherms differs little from that of the environment, being, in aquatic animals, less than 1°C. warmer than that of the water (D. L. Gunn, 1942). Amphibia, removed from the water, lose heat by evaporation and therefore have a body temperature lower than that of the air (except at 100 per cent humidity) (Adolph, 1932; Hall and Root, 1930). For practical purposes the body temperature of frogs in cages filled with sufficient water to cover the animals may be considered to be identical with that of the water. This situation does not exist, however, if the frogs are permitted to emerge from the water into a room at low relative humidity.

The metabolic rate of poikilotherms rises with increase in environmental temperature; the relationship is, however, non-linear (cf. Benedict, 1932; O'Connor and O'Donovan, 1942).

The highest body temperature tolerated by a given animal is greatly influenced by the time of exposure (cf. Bělehrádek, 1935). Unfortunately few authors have provided sufficient data to evaluate the importance of the time factor. Some frogs (*R. pipiens*) in a given group die within 24 hours in water at 33°C. (Chen et al., 1943a), and death occurs more rapidly at higher temperatures (Tigerstedt 1910; Kanitz, 1925). Many fish do not tolerate temperatures above 30°C. (Britton, 1924). Frogs and certain fish survive cooling to temperatures slightly below 0°C. (Cameron and Brownlee, 1914; Britton, 1924). The cause of death at both high and low temperatures is discussed by Heilbrunn (1943).

Homeotherms. Large homeotherms maintain a fairly constant body temperature at environmental temperatures from below 0°C. to above 40°C. The body temperature of mice and other small homeotherms, however, is in part determined by the temperature of the environment (Nielsen, 1938; Goldfeder, 1941; Chen et al., 1943b). For example, mice placed on a wire screen platform in individual glass cages at 15°C. showed a mean decrease in deep rectal temperature of 2.36°C. in one hour (G. J. Fuhrman, 1943). In mice, increased body temperature results from exposure to an environmental temperature of about 35°C. or above. For this reason attempts to investigate variations in drug

action in small animals at varied environmental temperatures may be complicated at both high and low extremes by changes in body temperature of the animals. In many studies at different environmental temperatures, body temperatures have not been reported. Anesthesia (Hemingway, 1940) and curare (Krogh, 1916) impair the temperature regulation of homeotherms to such an extent that the body temperature may then be manipulated by cooling or warming. Hibernating mammals appear to have a "labile" body temperature in the non-hibernating state (Benedict and Lee, 1938).

Acute hyperthermia in mammals results in increased metabolic rate, increased respiratory and heart rate and decrease in total peripheral resistance which may be more than compensated for by increased cardiac output (cf. Wiggers and Orias, 1932; Bazett, 1938). Death may occur from either respiratory or cardiac failure in the temperature range 40° to 45°C. (Kanitz, 1925; Marsh, 1930). The lethal level of hyperthermia in the fowl is about 47°C. (Randall, 1943).

Progressive general hypothermia (36° to 30°C.) in mammals results at first in increased heat production (from shivering and muscle rigidity) and hyperreflexia (Dill and Forbes, 1941; Fay and Smith, 1941; Grosse-Brockhoff and Schoedel, 1943a). At lower temperatures metabolic rate, heart rate, respiratory rate, cardiac output and arterial pressure all decrease. Reflexes are progressively abolished (Hamilton, 1937; cf. Libet et al., 1940). Lethal levels of hypothermia vary from about 20°C. in the dog (Grosse-Brockhoff and Schoedel, 1943b) to 13–16°C. in the rat (Crismon, 1944) and 10°C. in the rabbit (Ariel et al., 1943). Lower levels of hypothermia may perhaps be tolerated if some form of treatment precedes cooling; respiratory movements and cardiac activity were observed between 5° and 6°C. in fasted rats (Crismon and Fuhrman, 1946). The mechanism of death from hypothermia has been discussed by Grosse-Brockhoff and Schoedel (1943b) and by Crismon (1944). Hibernating mammals tolerate body temperatures as low as 0° to 3°C. (G. E. Johnson, 1931; Benedict and Lee, 1938).

Isolated tissues. Isolated vertebrate tissues survive over the approximate temperature range 0° to 42°C. if oxygen consumption and lactic acid production be taken as the criteria of survival (Fuhrman and Field, 1943; 1945; Field et al., 1944; G. J. Fuhrman, unpublished). The capacity for oxygen consumption of isolated frog muscle is impaired by a temperature approximately that necessary to produce heat paralysis in the intact animal (Brachet and Bremer, 1941; Feitelberg and Pick, 1942). Thermal reflex block in spinal centers of a frog nerve-muscle preparation occurred above 29°C. and below 14°C. (Ozorio de Almeida, 1943).

GENERAL COMPARISONS: DRUG ACTION IN HOMEOTHERMS AND IN POIKILOTHERMS. Inasmuch as there is a body temperature difference of almost 20°C. between mammals and frogs at room temperature, a comparison of pharmacodynamic effects in the two groups of animals yields data which depend in part upon the temperature difference. Such data are, in general, difficult to evaluate because of species differences.

Colchicine. Poisoning from seeds and galenical preparations of meadow saffron (*Colchicum autumnale*), and from eating the leaves as salad, led Jacobj (1890) to

undertake a more complete pharmacological investigation of the active alkaloid, colchicine, than had previously been made. He found that colchicine was far more toxic in mammals than in frogs. Earlier workers (cited by Jacobj) had found lethal doses to be extremely variable in both amphibia and mammals because of the use of impure and partially oxidized preparations. Jacobj prepared an oxidation product of colchicine, oxydicolchicine, and found that the action of this substance was qualitatively and quantitatively similar in both mammals and frogs. He concluded that colchicine itself is relatively non-toxic, and that it is oxidized in the body to the toxic oxydicolchicine. In the frog, at low temperatures, oxidation does not occur rapidly enough to produce toxic symptoms before excretion has occurred. Führer (1910) and Sanno (1911) found that the toxicity of colchicine in frogs (*R. esculenta*) was 400 to 500 times as great at 32°C. as at room temperature (about 20°C.). Frogs injected with colchicine and maintained at room temperature showed no toxic symptoms during several days, but died upon being warmed to 30–32°C.

In mammals the symptoms of colchicine poisoning are not observed until 3 to 6 hours after injection (Jacobj, 1890), and this latent period is not markedly shortened by increasing the dose. In frogs, at 20°C. death occurs after 14 days and at 30°C. after 48 hrs. (Führer and Breipohl, 1933).

In later experiments the fatal dose for frogs at 20°C. was found to be 1.3 mgm. per gm., while in mice it was 0.003 mgm. per gm. (Führer, 1932). The fatal dose for frogs at 30°C. was found to be 0.003 mgm. per gm. (Führer and Breipohl, 1933). The striking parallelism between the increase in toxicity in warm compared to cold frogs, and in mice compared to cold frogs, suggests that the temperature of the body is the chief factor influencing toxicity. The more rapid conversion of colchicine to the toxic oxidation product appears to be a satisfactory explanation for the results obtained.

Mussel poison. Frogs are much more resistant than are mammals toward the paralytic shell-fish poison from mussels (*Mytilus Californianus*) (Prinzmetal, Sommer and Leake, 1932).

Miscellaneous substances. Führer (1932) made an extensive series of comparisons of fatal doses of various drugs in frogs and mice. The temperature difference between the two groups was about 17°C. He found that methylguanidine, synephrine, phenol, caffeine, resorcinol and nupercaine were more toxic in the frog than in the mouse; the ratio of fatal doses (frog/mouse) ranged from 0.3 to 0.8. Histamine, procaine, chloral hydrate, pyrocatechin, hydroquinone and camphor were approximately equal in toxicity in the two groups of animals. Acetylcholine, aminopyrine, picrotoxin and sodium cyanide were more toxic in the mouse than in the frog, with ratios ranging from 2 to 6.5. Epinephrine was about 60 times as toxic in the mouse as in the frog.

A number of drugs produce central nervous system stimulation in homeotherms, but produce depression in frogs. Sodium 1,3-dimethyl-butyl-ethyl barbiturate produces convulsions in homeotherms (mice, rats, guinea pigs, rabbits, cats, dogs, monkeys) but only depression in poikilotherms (frogs and toads) (Swanson and Chen, 1939; Knoefel, 1945). Sodium 1-methyl-allyl-isobutyl

thiobarbiturate acts in a similar manner (Swanson, Fry and Reagan, 1945). Cocaine (J. A. Gunn, 1937) and harmine (Chen and Chen, 1932) likewise are convulsants in homeotherms and depressants in poikilotherms. Harmine is about twice as toxic in mice at an environmental temperature of 40°C. as at 20°C. (Chen et al., 1943b).

EFFECTS OF DRUGS DURING HIBERNATION. The body temperature of mammals during hibernation falls to a level only slightly above that of the environment. Differences observed in the effect of drugs in animals during hibernation and the effects in animals at body temperatures of 37–39°C. may be in part accounted for on the basis of the difference in temperature. Such a comparison is, however, complicated by physiological changes occurring during hibernation, e.g., acidosis (Stormont et al., 1939).

In 1899 Koeninck reported results of the subcutaneous administration of strychnine, picrotoxin, muscarine, pilocarpine, caffeine and apomorphine to a few hibernating bats (*Nannugo Pipistrellus*) caught in the cellar of the Marburg castle. At an environmental temperature of 6–7°C. the effects were somewhat slower in appearing than in non-hibernating animals, but the actions were qualitatively similar. Koeninck reported that hibernating bats tolerated larger doses of tetanus toxin than did bats which were not hibernating. His data for the other substances are not sufficient to permit quantitative comparison with animals at normal body temperatures. Hausmann (1906), using larger numbers of bats (*Vesperugo noctula*) found that colchicine, saponin, tannin and abrin produced death much more rapidly in bats which were active than in hibernating bats. Larger doses of colchicine were tolerated by the hibernating animals. Marmots injected with tetanus toxin showed no symptoms of tetanus while hibernating, but developed tetanus upon awakening (Billinger, 1896).

Marmots during hibernation were about as sensitive as non-hibernating animals to the effects of cobra venom, diphtheria and tetanus toxin and inoculation with parasites; the effects were slower in developing in the hibernating animals (Blanchard, 1903).

Recently Pfeiffer, Foster and Slight (1939) carried out a more extensive study of the effects of a number of analeptic drugs on the thirteen-lined ground squirrel (*Citellus tridecemlineatus*). Active animals were at a body temperature of 32–40°C.; while hibernating the body temperature of the squirrels was 4–10°C. Lethal and convulsant doses were determined in the awake and hibernating animals for ephedrine, neosynephrine, epinephrine, benzedrine, cocaine, picrotoxin, metrazol, coramine, caffeine with sodium benzoate and strychnine. It was found that caffeine and strychnine were 8 and 10 times, respectively, more toxic in the hibernating animals than in active ones, probably as a result of the high blood pCO_2 in hibernation. Asphyxia renders normal animals more susceptible to strychnine convulsions. Epinephrine produced convulsions in hibernating animals only. Clonic convulsions, produced by coramine for example, were well tolerated by the hibernating animals, and persisted for 2–3 days. Convulsions produced by strychnine were poorly tolerated.

The effect of various drugs in awakening animals from the hibernating state,

and their relative efficiency (cf. Pfeiffer et al., 1939) are not within the scope of the present review.

DIGITALIS AND RELATED SUBSTANCES. *Poikilotherms.* Ever since attempts were made early in this century to use frogs for the bioassay of digitalis, various investigators have recognized that season and temperature are among the factors influencing the sensitivity of frogs to the cardiac action of the digitalis glycosides. In the early work, part of the "seasonal variation" was undoubtedly due to temperature, but later results indicate a seasonal variation independent of assay temperature. Focke (1907) was one of the first actually to demonstrate the influence of temperature upon the sensitivity of frogs. He found that by warming frogs in winter he could increase their sensitivity to about that of summer frogs. In the next few years Edmunds and Hale (1908), Hale (1911), Ginzberg and Hohlberg (1913), Vanderkleed and Pittinger (1913) and Gottlieb (1914) all recognized the importance of temperature in the frog method of bioassay of digitalis.

Baker (1912), using the 1 hour frog method (cf. Munch, 1931 for this and other methods of bioassay), found that from 10° to 30°C. there was a progressive decrease in the minimum systolic dose (MSD) of both ouabain and digitalis. Haskell (1914), using Houghton's lethal method, obtained similar results. Both found that the difference in toxicity of ouabain with change in temperature was greater than that of digitalis. The MSD of digitoxin, determined by the 1 hour frog method, was found to be 13, 9 and 8 mgm. per kgm. at 10°C., 20°C. and 30°C. respectively (Roth, 1916). Weese (1936, p. 20) gives lethal doses for frogs of digitoxin, gitalin, ouabain and strophanthin-k, determined by Houghton's lethal method, at 15°, 20° and 25°C. which also clearly demonstrate increased toxicity at higher temperatures. Goldfish died more rapidly in warm digitalis solutions than in cold ones (Pittinger and Vanderkleed, 1915). Sollmann et al. (1915) determined the MSD of ouabain by the 1 hour frog method over the range 3° to 30°C., and found that the toxicity increases markedly with increase in temperature. At 3–4°C. the MSD was 26.1 mgm. per kgm., while at 26°C. it was only 0.3 mgm. per kgm. The increase in toxicity per degree of temperature increase was much greater at the lower than at the higher temperatures. Hirschfelder et al. (1920) extended these observations on frogs as high as 36°C. and found increased toxicity of digitalis from 30° to 36°C. Higher temperatures cannot be studied using frogs because of excessive mortality of controls at these temperatures. Sollmann et al. (1915), Roth (1916) and Defandorf (1933) recognized that differences in rate of absorption at different temperatures complicated the interpretation of the results obtained on intact frogs. Smith and McClosky (1925) circumvented the complicating effects of temperature upon absorption rate by intravenous injection of digitalis in frogs. They found that at 15°, 20°, 25°, 30° and 37°C. (body temperature) the MSD for an international digitalis leaf powder was 740, 400, 300, 250 and 80 mgm. per kgm. respectively. Thus, although rate of absorption may vary with temperature, a large part of the difference found is due to a more direct effect of temperature upon the action of digitalis.

Curves relating temperature to MSD for ouabain (Baker, 1912; Sollmann et al.,

1915) and for digitalis (Smith and McClosky, 1925) are similar in shape. On the basis of temperature effects there is therefore no reason to believe that the fundamental action of these two substances is different, as had been inferred from a comparison of their effects following injection into the lymph sac (cf. Smith and McClosky, 1925). Differences in rate of absorption of the two drugs apparently led to the differences obtained by Baker (1912).

Chen et al. (1943a) have recently determined the effect of temperature upon the LD_{50} of two other digitalis-like substances: cymarin, the active glycoside from Canadian Hemp (*Apocynum*), and coumingine hydrochloride, an alkaloid from *Erythrophloeum couminga*. They were studied in *R. pipiens* over the temperature range 13° to 33°C. Coumingine hydrochloride was approximately 5 times as potent at 33°C. as at 13°C., while cymarin was about twice as active at the higher temperature.

Isolated heart. Trendelenburg (1909) noted that with strophanthin-k and antiarin (from *Antiaris toxicaria*, the Upas tree of Java) the time required to produce systolic standstill of the isolated (Straub) heart increased as the temperature was lowered. This observation has been confirmed and the data extended to other drugs of the digitalis series. This work was done chiefly by: Weizsäcker (1913) and Sollmann et al. (1915) using ouabain; Fischer (1928) using digitoxin and gitoxigenin; Gander (1932) using gitalin and its aglycone gitaligenin.

H. Fischer (cf. Weese, 1936, p. 127) in a series of studies of digitalis-like substances on the isolated frog heart, separated the binding of digitalis by the heart muscle into several phases: I—membrane phase, II—fixation phase, III—action phase. The influence of temperature upon phases II and III was studied in order to determine whether or not these were chemical in nature. Fixation occurs more rapidly at higher temperatures (v. Issekutz, 1914; Sollmann et al., 1915). The Q_{10} for the process of fixation (phase II) lies between 2.3 and 3.8 for digitoxin (Fischer, 1928) and gitalin (Gander, 1932). The Q_{10} for the rate of action (phase III) is of the same general magnitude (2–3) for digitoxin, gitoxigenin (Fischer, 1928), gitalin and gitaligenin (Gander, 1932). On the basis of a Q_{10} of 2 to 3 the processes of fixation and of action in producing systolic standstill are therefore believed to be chemical, rather than physical, in nature.

The Q_{10} of the time required for the production of systolic standstill of the frog heart by ouabain varied from 6.1 to 17 (Sollmann et al., 1915). The activity was dependent upon both temperature and heart rate; the high Q_{10} values were accounted for on the basis of a summation of the effect of temperature upon both processes. Weizsäcker (1913), however, maintained the heart rate constant, and obtained data showing that the rate of action of digitalis increases with increase in temperature. Fischer (1928) found the action of temperature in increasing the rate of action of digitoxin largely independent of the effect of temperature on the heart rate and amplitude. The effect of temperature on the rate of fixation and of action was not dependent upon the calcium content of the Ringer solution (Fischer, 1928).

The concentration of drug necessary to produce systolic standstill *in a given time* also decreases with increase in temperature for strophanthin-k (Trendelen-

burg, 1909), ouabain (Sollmann et al., 1915), digitoxin, gitoxigenin (Fischer, 1928), gitalin and gitaligenin (Gander, 1932). Likewise, the lowest concentration of drug which will produce systolic standstill (Grenzkoncentration) is lower at higher temperatures, not only in the perfused frog heart, but also when strips of frog heart sinus are used (Mansfeld and Horn, 1928). On the embryonic chick heart Paff and Johnson (1938) found that a given concentration of ouabain produced A-V block more rapidly at 38°C. than at 28° or 20°C. Block produced with 1:600,000 ouabain at 38°C. could be made to disappear by reducing the temperature to about 20°C.

In the isolated perfused rabbit heart, ouabain produced complete arrest more rapidly at higher temperatures (J. W. C. Gunn, 1914). At a given temperature and a given strength of ouabain solution, differences in rate of action were obtained by varying the rate of coronary perfusion. Gunn believed variation in rate of blood flow to be responsible for the temperature effects. However, the effect of temperature was still evident in the figures given when the rate of coronary perfusion was constant: 1:500,000 ouabain, 218 cc. stopped the heart in 28 min. at 30°C.; 220 cc. stopped the heart in 21 min. at 40°C.

In general the results obtained on the isolated perfused heart are in agreement with those obtained on intact frogs. In both types of preparations the intensity of action of cardiac glycosides increases with increase in temperature.

Homeotherms. The effect of temperature upon the action of digitalis in homeotherms has been extensively studied only during hyperthermia. In man, and in some experimental work where fever was produced by toxins or infections, the effect of temperature may be complicated by the action of toxins on the heart (cf. Weese, 1936, p. 233; McGuigan, 1938).

Jamieson (1915) reported the same lethal dose of ouabain in cats infected with pneumonia as in normal animals, but the body temperature rise in the infected animals was usually less than 1°C. Herzog and Schwartz (1930) found that the lethal dose of strophanthin was greater in cats and rabbits with fever than in normal animals. The isolated heart of the animals with fever was stopped with approximately the same dose required to stop the heart of normal animals, so that the authors concluded that the lower sensitivity of the intact animals with fever was of extra-cardiac origin. Weese (1932) however, reported a decreased sensitivity of the isolated heart to strophanthin only when the heart of an animal with fever was perfused with blood from animals with fever. He attributed the difference to cardiac and humoral changes resulting from the fever.

Data in which the results are not complicated by the presence of toxins do not confirm the results of Herzog and Schwartz. The lethal doses of tincture of digitalis, in anesthetized cats warmed by immersion in water, were found to be 0.94, 0.78, 0.59 and 0.375 cc. per kgm. at body temperatures of 38, 41, 42 and 43°C. respectively (Hirschfelder et al., 1920). McGuigan (1938) administered digitalis in divided doses to dogs under barbiturate anesthesia. The MLD in control animals was 123 mgm. per kgm., while in animals in which hyperthermia (average increase in temperature = 3.25°C.) was induced by the administration of dinitrophenol, the MLD was about 73 per cent of the control. In further experi-

ments, in which hyperthermia was induced by diathermy, the MLD at 40°, 41°, 42°, 43° and 44°C. amounted to 82, 81, 66, 61 and 50 per cent of the control respectively. Slowing of the heart rate was produced by digitalis during hyperthermia.

The therapeutic implications of these experiments are evident. The tachycardia in fever is chiefly the result of the direct effect of temperature upon the heart muscle. Although slowing of the heart may be produced by digitalis, this may be obscured by the speeding of the heart resulting from rise in temperature. Larger doses of digitalis cannot, however, be tolerated, since for each 1°C. rise in temperature the toxicity of digitalis is increased 10 to 15 per cent. Both Hirschfelder et al. (1920) and McGuigan (1938) caution against the use of large doses of digitalis in fever.

Comparisons between homeotherms and poikilotherms. The very great difference between the fatal dose of digitalis glycosides and pharmacologically related substances in the frog and in the cat or dog is evident from the foregoing discussion. McGuigan (1938) cites fatal doses of digitalis in the dog as 100 mgm. per kgm. and in the frog as 600 mgm. per kgm., and attributes much of this difference to a difference in body temperature. Führer (1931) reported that ouabain and cymarin were more toxic in the frog than in the mouse, but this discrepancy is to be accounted for on the basis of the well known resistance of mice (and rats) to digitalis-like substances (cf. Chen and Rose, 1942). Cats, rabbits and guinea pigs (Chen and Rose, 1942) are much more susceptible to the action of cymarin, ouabain and coumingine hydrochloride than are Leopard frogs (*R. pipiens*) or tree frogs (*Hyla cinerea cinerea*) (Blair, Hargreaves and Chen, 1940). Since the frogs were studied at 20°C., there was a body temperature difference of about 17°C. between the homeotherms and poikilotherms. This may account for a large part of the difference in sensitivity. Slow absorption from the lymph sac in frogs may also be important. Smith and McClosky (1925) determined the MLD of digitalis in the cat to be 90 mgm. per kgm.; by intravenous administration to frogs (where rate of absorption is not a factor) the MLD was found to be 80 mgm. per kgm. at 37°C. It would be preferable to make such a comparison at a body temperature of about 30°C. for both animals, since frogs tolerate 37°C. for only brief periods. The close agreement between the two fatal doses at 37°C. suggests that temperature is chiefly responsible for the differences in fatal dose found between homeotherms at 37°C. and poikilotherms at about 20°C.

CENTRAL NERVOUS SYSTEM STIMULANTS. *Poikilotherms.* Kunde (1860) observed that if frogs were injected with a strychnine solution and placed in water at 1°, 16° and 31°C., those at 31°C. developed convulsions first. Strychnine convulsions, produced in frogs at room temperature, disappeared upon placing the animals in water at 37°C. (Foster, 1873). Blume (1930) found the rate of action of strychnine in fish (*Carassius vulgaris*) to increase with increasing water temperature.

Githens (1913) made a more thorough study of the influence of temperature upon the action of strychnine in frogs. With a dose of 0.6 mgm. per kgm., convulsions occurred in frogs at all temperatures from 5° to 30°C., while with a dose

of 0.3 mgm. per kgm., convulsions occurred only at 5°C. and above 24°C. These optimum temperatures correspond to the optimum temperatures at which reflex activity of the spinal cord of frogs (*R. temporaria* and *R. esculenta*) is reported to occur (Storm van Leeuwen and van der Made, 1916).

Schlomovitz and Chase (1916) and Schlomovitz and Machlis (1926) studied the influence of temperature upon the time of onset of convulsions following strychnine administration to frogs (*R. pipiens*). Over a temperature range of 1° to 36°C. the time between injection (into the dorsal lymph sac) and the appearance of convulsions decreased progressively with increase in temperature. If the heart rate was varied independently of body temperature, the time between injection and convulsions was only very slightly changed, so that the results were attributed to other causes than increase in heart rate with increase in temperature. It is likely that at high temperatures an increased rate of absorption resulted from increased circulation in the region of injection.

Lambruschini (1938) and Penhos (1944) found the LD_{50} for strychnine sulfate in the toad (*Bufo arenarum* Hensel) to decrease with increase in temperature: at 5°, 18° and 35°C. the LD_{50} was 120, 50 and 2.5 mgm. per kgm. respectively (Penhos). At 5°C. the interval between injection and appearance of the convulsions with the LD_0 was 20 minutes, while at 35°C. it was 6 minutes. Führer and Breipohl (1933) reported a lower MLD for strychnine in the frog at 30°C. than at 20°C.

Gast (1943) found a lower fatal dose of strychnine nitrate, given to frogs (*R. temporaria* and *R. esculenta*) by the ventral lymph sac, at 29°C. than at lower temperatures. The time from injection to the appearance of convulsions was 7 hours at 2°C. and 20–40 minutes at 29°C. This difference, however, was reported to disappear upon intravenous injection, and convulsions then appeared immediately in both cold and warm frogs. It thus seems probable that differences in rate of action which have been found after injection into the lymph sac are due to differences in absorption rates at different temperatures. Gast found the time between injection and recovery from convulsions to be dependent upon the temperature. For example, with 0.75 mgm. per kgm., recovery occurred after 40, 16, 6 and 3 hours at 1°C., 10°, 20° and 29°C. In nephrectomized frogs the same relationship between recovery-time and temperature was found as in normal frogs so that destruction of strychnine by the tissues was thought to be the most important mechanism of detoxification. Penhos (1944) found a slightly greater mortality for strychnine in toads after ligation of the ureters.

Strychnine oxide is not effective in producing convulsions until it is reduced to strychnine in the body. In frogs the latent period between injection and appearance of convulsions with 20 mgm. per kgm. was found to be 2–3 days, 1½ days, 9–12 hrs. and 1–2 hrs. at temperatures of 1°, 10°, 20° and 29°C. (Gast, 1943). This latent period was not altered by intravenous injection. As with strychnine, the time for recovery following administration of strychnine oxide was longer at low temperatures than at high ones. Gast and others reported that at 1–2°C. the convulsions produced by strychnine differed from those at 30°C. by their

longer duration, heightened tonus between convulsions and lack of "blitzartig" character.

These differences probably result from the effects of cold upon the activity of the nervous system rather than from a different type of action of strychnine at the lower temperatures. Cold enhances the reflex activity of the spinal cord in mammals (Grundfest, 1941). Using single frog axons, Schoepfle and Erlanger (1941) found that cold lowered the excitability, and increased the height and duration of the spike, but the descent was prolonged more than the ascent. The amplitude of spontaneous oscillations in excitability was increased by cold and by strychnine (Erlanger et al., 1941).

Other central nervous system stimulants have not received the attention that has been accorded strychnine. Luchsinger (1878) reported that with picrotoxin the time from injection to appearance of convulsions in frogs was greater at lower temperatures, from 0° to 32°C. The fatal dose of picrotoxin in frogs was found to be 9 mgm. per kgm. at 30°C. and 30 mgm. per kgm. at 20°C. (Fühner and Breipohl, 1933). The time between injection and death was about 7 times as long at 20°C. as at 30°C.

In the toad (*Bufo arenarum* Hensel) caffeine, injected into the ventral lymph sac, was found to be more toxic at 4°C. than at 33°C. (Günther and Odoriz, 1944). At 4°C., in the toad, caffeine produces a marked decrease in urine volume (in contrast to diuresis at 36°C.) and more intense hypertonia than at higher temperature (Günther and Odoriz, 1944; 1945). The protective action of the higher temperature could not be explained on the basis of increased excretion, since only about 10 per cent of the total dose was excreted by the kidney at 36°C. As the authors suggest, the lower toxicity of caffeine at high temperatures is probably due to the more rapid detoxification (demethylation) of the drug with increase in temperature.

Homeotherms. In mice, cooled to 20°C. body temperature, Leser et al. (1940) found that survival after 3 mgm. per kgm. strychnine sulfate was prolonged 3.5 times compared to uncooled controls. Mortality was greater in the cooled animals (100 per cent) than in controls (72 per cent). Gast (1943) cites unpublished data indicating increased toxicity of strychnine in hypothermic mammals.

Cats anesthetized with barbital were cooled to a body temperature of 20–23°C., and the action of analeptics injected intravenously compared with the action in anesthetized controls at 38°C. (Stormont and Hook, 1941). In the hypothermic animals the lethal dose of picrotoxin was about twice that in normal animals; the toxicity of metrazol was unchanged; and strychnine was slightly more toxic than in controls.

The toxicity of metrazol in mice was not significantly different at a body temperature of 20°C. from that at 37°C. (Leser et al., 1940). The time between intraperitoneal injection and appearance of convulsions was 11 times as long at 20°C. as at 37°C.

Coramine and lobeline were more toxic in dogs under urethane-morphine anesthesia at body temperatures of 30°C. and below than at 38°C. (Grosse-Brockhoff and Schoedel, 1943c). Doses which were active at normal body temperature

were often fatal in the hypothermic animals, and smaller doses were without effect. The increased toxicity during hypothermia was attributed to an increased vagal action (see acetylcholine). Evidence of increased toxicity of analeptics in hypothermic animals has also been obtained by Jarisch (1943). These and other analeptic drugs were ineffective in "awakening" animals during hypothermia (Grosse-Brockhoff and Schoedel, 1943c).

Caffeine with sodium benzoate, injected subcutaneously in mice in a dose of 178 mgm. per kgm., was less toxic at an environmental temperature of 30°C. than at 10°C. (Günther and Navarro, 1945).

CENTRAL NERVOUS SYSTEM DEPRESSANTS. *Poikilotherms and isolated preparations.* One of the experimental findings which forms the basis of the Meyer-Overton theory of narcosis is the parallel change in partition coefficient and narcotic action with change in temperature reported by H. H. Meyer in 1901. Meyer found, using tadpoles, that a rise in temperature (from 3° to 30°C.) increases the oil/water partition coefficient and the narcotic action of chloral hydrate, ethyl alcohol and acetone; on the other hand, a rise in temperature decreases both the partition coefficient and narcotic action of salicylamide, benzamide and monoacetin. Similar experiments have been performed by others. Although some of the results are conflicting, the weight of the evidence confirms and strengthens Meyer's results. Henderson (1930) has reviewed this subject and cited the more pertinent papers; references to the influence of temperature upon alteration of surface tension by narcotics are also given. Recently McElroy (1943) has reported the effect of temperature upon narcotic action (in the case of luminous bacteria) and has discussed the relationship of lipoid solubility and enzyme denaturation to narcosis.

The rate of action of ethyl alcohol and chloroform as a function of temperature was investigated by Veley and Waller (1910) on isolated frog muscle. The data were found to yield straight lines when the logarithm of the decrement of time for complete inhibition of contraction was plotted as a function of the logarithm of the increment of absolute temperature (cf. Bělehrádek, 1935). The Q_{10} for the inhibition of muscle contraction was 2.04 for alcohol, 1.63 for chloroform and 2.0 for ether (Waller, 1909).

The temperature characteristic (μ) for oxygen consumption of the newt under chloretone anesthesia was the same as in unanesthetized animals; it was altered under pentobarbital anesthesia (Gordon and Pomerat, 1942).

Toads at 3°C. tolerate doses of morphine sulfate which are fatal at 20° or 30°C. (Lambruschini, 1938). In general the fatal dose was found to decrease with increase in temperature.

The effect of temperature upon the toxicity of some other hypnotics in frogs was reported by Richards (1941). The LD_{50} for paraldehyde was 4.4 grams per kgm. at 10°C., 3.9 gms. per kgm. at 20°C. and 2.5 gms. per kgm. at 30°C. The LD_{50} for pentothal and pentobarbital decreased with increase in temperature at 20°, 30° and 35°C. However, for these two drugs, the LD_{50} was lower at 10° than at 20°C.

Homeotherms. Cameron (1939) reported that rats anesthetized with a given

dose of pentobarbital died if placed in a draft during November, but invariably recovered if kept warm. Similar observations had been made earlier. L. Hermann (1867) found that rabbits tolerated alcohol better if they were warmed than if they were allowed to cool spontaneously at room temperature. Brunton (1874, 1885) states that death could be prevented in animals poisoned with chloral by warming. In the above experiments the animals which were not warmed unquestionably suffered considerable fall in body temperature, since anesthetics suppress reflex responses to cold (Hemingway, 1940).

The newborn of most species of mammals (with the outstanding exception of the guinea pig) are more resistant to anoxia than the adults of the species (cf. Windle, 1943). This is true whether the animals breathe nitrogen or other inert gases, or whether gaseous anesthetics such as nitrous oxide or cyclopropane are used (Fazekas, Alexander and Himwich, 1941). These authors demonstrated that elevation of environmental temperature shortens the survival time, so that the poikilothermia of the infant is one of the factors concerned in the better survival. Barrows (1933) found that the survival time of mice in ether vapor decreased with increasing age of the animals, and called attention to the relationship between survival and the development of temperature regulation in mice as demonstrated by Sumner (1913). More recently Barrows and Dodds (1942) determined the survival time of mice of different ages in ether or chloroform vapor at environmental temperatures of 22°, 28°, 35° and 40°C. For a given age up to about three weeks the survival time increases with a decrease in environmental temperature, and, since mice of this age are essentially poikilotherms, survival time increases with a decrease in body temperature as well.

In rats 20 mgm. per kgm. morphine sulfate was fatal in some animals exposed to an environmental temperature of 3°C., but not in animals at room temperature (Herrmann, 1941). Fall in body temperature to 23°C. in 6 hours at 3°C. occurred, so that it is probable that hypothermia was a contributing factor in the mortality. Similar results were obtained with paraldehyde and pentobarbital.

In mice cooled to 20°C., Leser et al. (1940) found the survival time with 350 mgm. per kgm. morphine sulfate to be increased in the cooled animals and the toxicity slightly, but probably not significantly, increased.

Raventos (1938) carried out the most complete study of the effect of environmental temperature yet reported on the action of barbiturates. The duration of anesthesia produced in mice by intraperitoneal administration of 100 mgm. per kgm. sodium evipal was 2.5 times as long at 20° as at 30°C. and was shorter at 40° than at 30°C. The HD_{50} (median hypnotic dose) for the same drug was 39 mgm. per kgm. at 30° and 45 mgm. per kgm. at 20°C.; the LD_{50} was the same at both temperatures. With sodium phenobarbital the HD_{50} was 105 mgm. per kgm. at 30° and 90 mgm. per kgm. at 20°C.; the LD_{50} decreased from 234 mgm. per kgm. at 30° to 162 mgm. per kgm. at 20°C. The difference in the effect of temperature upon the lethal doses of the two barbiturates was accounted for on the basis of a difference in time required to produce death. With evipal death occurred soon after injection, before body temperature of the mice was reduced; with phenobarbital death occurred 3 to 24 hours after injection at a time when

the body temperature of the animals reached that of the environment, so that hypothermia was a contributory cause of death in the case of animals given phenobarbital and exposed to 20°C. Raventos emphasizes that part of the discrepancies which have been previously reported in both lethal and hypnotic doses of barbiturates are due to failure to control environmental temperature.

Gaylord and Hodge (1944) reported that in both normal and castrate female rats given 30 mgm. per kgm. pentobarbital subcutaneously at 13°, 23° and 37°C., the duration of sleep increased with decrease in environmental temperature.

The reports of Cameron (1939) and Gaylord and Hodge (1944) using pentobarbital, and of Raventos (1938) using evipal, indicate that rats and mice sleep longer with a given dose of the barbiturate at lower environmental temperatures. Since, in anesthetized animals (particularly in mice), the body temperature falls rapidly toward that of the environment, this difference in sleeping time may be due to a decreased rate of detoxification of these barbiturates at the lower temperatures. Since it is known that all common barbiturates, with the exception of sodium barbital, are largely detoxified in the body (Masson and Beland, 1945), it would be desirable to compare the sleeping time of mice at different temperatures using barbital and one of the rapid-acting group such as pentobarbital. This was done in this laboratory (Fuhrman, 1946). Forty-six white mice weighing 14–30 grams were injected intraperitoneally with 37.5 mgm. per kgm. sodium pentobarbital or 290 mgm. per kgm. sodium barbital. They were maintained at room temperature (25°C.) until anesthesia developed, then half of each group were cooled to a body temperature of 27°C., and half were warmed to a body temperature of 37°C. With sodium pentobarbital the mean sleeping time at 37°C. was 17.5 minutes, while at 27°C. it was 61.8 minutes. The difference between the means was significant ($P < 0.0001$). With sodium barbital the mean sleeping time at 27°C. was 331.5 minutes, while at 37°C. it was 314.5 minutes. The difference between these means was not significant ($P < 0.2$).

These data are in agreement with the hypothesis that for a drug which is chemically detoxified by the tissues (pentobarbital) the action is prolonged by hypothermia, while for a drug which is not detoxified (barbital) the duration of action may not be influenced by low body temperatures.

Ethyl alcohol metabolism. The rate of alcohol metabolism in the frog (*R. temporaria*) increases with increase in temperature, yielding a Q_{10} of 1.97 over the range 1°C. to 29°C. (Nicloux, 1931). In 24 hours 14.7 per cent of the alcohol administered was metabolized at 1°C., while 97 per cent was destroyed at 29°C. These data are consistent with the view that alcohol is oxidized enzymatically (chiefly in the liver) and that reduction in the temperature at which the oxidation occurs results in a slowing of the rate of oxidation.

In homeotherms the rate of metabolism of alcohol is higher in small animals with high metabolic rates than in larger animals (LeBreton, Nicloux and Shaeffer, 1935; Newman and Lehman, 1937). In a given species, however, attempts to increase the rate of alcohol metabolism by increasing the metabolic rate with thyroxin (Widmark, 1935; Harger and Hulpieu, 1935), dinitrophenol (Newman and Tainter, 1936) or by exposure to a cold environment (LeBreton, 1934) did

not succeed in significantly altering the rate of combustion of alcohol. By elevation of body temperature by means of diathermy, slight increases in rate of metabolism of alcohol have been reported (Fleming and Reynolds, 1935; Ewing, 1940). The increase in temperature obtained was so small that changes in rate of alcohol metabolism are difficult to demonstrate. Fuhrman and Fuhrman (unpublished) determined the rate of metabolism of alcohol in rabbits at body temperatures of 38°, 30°, 27.5° and 25°C. after intravenous injection of 0.78 grams per kgm. alcohol. The rate of combustion of alcohol in all of the hypothermic animals was lower than that at normal body temperatures; the rate of combustion was 100 mgm. per kgm. per hour at 25°C. compared to 156 mgm. per kgm. per hour at 38°C. These data, taken together with those demonstrating failure to increase the rate of combustion of alcohol by an increase in metabolic rate, show that in homeotherms, as well as in poikilotherms, the rate of alcohol metabolism is dependent upon body temperature.

Dinitrophenol. The calorigenic effect of 2–4 dinitrophenol has been reported to be less apparent in homeotherms at low environmental temperature than at a temperature in the neighborhood of thermal neutrality (Magne, Mayer and Plantefol, 1932; Giaja and Dmitrijevic, 1933; Tainter, 1934). At low environmental temperatures animals injected with large doses of dinitrophenol may become hypothermic. Tainter (1934) found that the body temperature of rats given 30 mgm. per kgm. dinitrophenol fell 4.1°C. in 4 hrs. at 2°C. air temperature, compared to 1.6°C. fall in controls. G. J. Fuhrman et al. (1943), using mice at 6°C. found that 50 mgm. per kgm. resulted in a fall in body temperature to 21–22°C. and death of the animals within one hour.

G. J. Fuhrman et al. also reported the LD_{50} of 2–4 dinitrophenol administered subcutaneously in mice at different environmental temperatures to be 35.7 mgm. per kgm. at 6°C., 30.9 mgm. per kgm. at 25°C., and much lower yet at 40°C. Body temperatures of the mice fell at 6°C. and 25°C.

In order to elucidate the influence of cold upon the calorigenic effect of dinitrophenol, Hall et al. (1937) investigated its effect upon oxygen consumption and shivering in anesthetized cats rendered hypothermic by immersion in cold water. In the hypothermic cats dinitrophenol greatly decreased or abolished shivering in spite of the continuation of conditions normally evoking shivering. Thus dinitrophenol depresses the mechanism for chemical defense against cold. When allowance was made for the oxygen cost of shivering it was shown that the calorigenic action of dinitrophenol on the cat was augmented by hypothermia, and that the intensity of the calorigenic effect was greatest over the body temperature range 33° to 35°C. Subsequent work of Hall and Chamberlin (1937) demonstrated a synergic calorigenic action of dinitrophenol and epinephrine when the latter was infused intravenously at a physiological rate. Epinephrine secretion during hypothermia was suggested as a major factor in enhancement of dinitrophenol action in hypothermic animals.

Hall et al. (1937) suggested that the depression of shivering by dinitrophenol was of central origin and that "should dinitrophenol increase the local cellular metabolism of the portion of the gray matter constituting the heat regulatory

center, to a sufficient degree, its threshold of excitation by cooled blood or by cold receptor impulses would be increased. It would result that the center would be thermostatically adjusted to regulate at a lower body temperature than normal." Evidence for such a hypothesis was provided by Fuhrman and Field (1942) who found that the maximum stimulation of oxygen consumption of excised rat cerebral cortex by dinitrophenol occurred at 32°C. Thus in excised rat brain and in the intact cat the maximum stimulation of oxygen consumption by dinitrophenol occurs at approximately the same temperature. In other excised tissues the temperature at which maximum stimulation occurs is different; 22°C. for rat kidney cortex (Fuhrman and Field, 1942) and 35°C. for rat skeletal muscle (Hollinger, 1944–45).

HORMONES. *Epinephrine.* Langlois (1897) observed that the increase in heart rate of the turtle, produced by epinephrine, persisted longer at 15°C. than at 37°C. He then found that the response to epinephrine injection in mammals was prolonged in an animal cooled to 35°C. (Langlois, 1898). A similar prolongation was noted by Lewandowsky (1899) for the dilatation of the cat's pupil. Elliott (1905) sought an answer to the question of whether this prolongation was due to a delayed disappearance of epinephrine or to alteration of the muscle as a result of the reduced temperature. He concluded, from experiments with both local and general cooling, that cooling increases the duration of contraction of a muscle, but that the contraction produced by epinephrine administration is prolonged beyond that of the cooled controls. McGuigan (1938) reported the increase in blood pressure of dogs in response to epinephrine to be less at temperatures above normal, but did not attribute the change in response to a more rapid destruction of epinephrine at the higher temperatures.

The effect of temperature upon the response of the nictitating membrane of the cat to epinephrine was investigated by Fuhrman et al. (1944). They found that, for a given dose of epinephrine, the response of the nictitating membrane became progressively greater as the body temperature was reduced. Over the temperature range 12.9°C. to 42.3°C. the logarithm of the duration of contraction of the nictitating membrane was a linear function of the temperature. Regional warming of the liver of hypothermic cats (by means of long wave diathermy) without change in either rectal temperature or temperature of the nictitating membrane, shortened the response of the membrane to a given dose of epinephrine. At reduced levels of body temperature ventricular fibrillation was produced by doses of epinephrine which produced no evidence of cardiac irregularity at higher temperatures. These results were interpreted to mean that the enzymatic detoxification of epinephrine is slower at low temperatures; a given dose therefore produces a longer and more intense action during hypothermia than at normal body temperature. In agreement with this view the fatal dose of epinephrine in frogs is greater at 20°C. than at 30°C. (Führer and Breipohl, 1933).

In contrast to these findings on intact animals, a number of experiments on isolated heart and blood vessels have shown that the maximum activity of epinephrine is exhibited at about 37°C. On the isolated rabbit heart the activity

of epinephrine was reported to be greater at 37° than at 18°C. (Panella, 1907). The amplitude of contraction of the perfused frog heart was increased more at temperatures above 18°C. than below (Sprinceana, 1915). The rate response of the perfused frog heart to epinephrine increased with increase in temperature (Barlow and Sollmann, 1926). The optimum temperature for action of epinephrine upon perfused intestinal blood vessels was 37–39°C. (Kosuge, 1935). All of these experiments differ from those reported above in that no mechanism exists for epinephrine detoxification, since the liver is not a part of the perfusion system. In the intact animal, then, the rate of destruction of epinephrine appears to be the principal factor determining its intensity of action.

Insulin. Poikilotherms. Krogh and Rehberg (cf. Macleod, 1926; Hemmingsen, 1925) observed that frogs injected with insulin developed convulsions only after 4 to 5 days. Further investigation of the rate of action of insulin in poikilotherms has shown that the length of the latent period increases with decrease in temperature.

Frogs given 0.45 to 3 units of insulin developed convulsions at 30°C. within 14 hours and all died; at lower temperatures death did not result, and convulsions developed at 25°C. in 24–27 hours, at 20°C. in 43–49 hours, at 15°C. in 60–70 hours and at 7°C. in 120–144 hours (Huxley and Fulton, 1924). Extrapolation to 37°C. indicated that frogs should develop convulsions in $1\frac{1}{2}$ to $2\frac{1}{2}$ hours at that temperature; this time is not greatly different from that found in rabbits. Olmsted (1924) confirmed these observations in frogs and later found in toads that convulsions occurred 18, 24 and 36 hours after insulin injection at 30°, 25° and 18°C. respectively (Olmsted, 1926). In the frog, in which blood sugar was elevated by exposure to high temperature (Olmsted, 1924), and in the sculpin (*Myoxocephalus*), in which the normal blood sugar is low (McCormick and Macleod, 1925), insulin administration resulted in little change in blood sugar. In other poikilotherms, including frogs, turtles, snakes, fish and the Cayman, insulin produced hypoglycemia after several hours (Sordelli, Houssay and Mazzocco, 1923; Houssay and Rietti, 1924; Mann, Bollmann and Magath, 1924; Hemmingsen, 1925; Barlow, Vigor and Peck, 1931). In the brown trout (*Salmo faris*) at 4°C., and the menhaden (*Brevoortia tyrannus*) and scup (*Stenotomus Crysops*) at 19.5°C., insulin produced a marked fall in blood sugar, and convulsions developed approximately 24–60 hours, 23 hours and 7 hours respectively, after injection. In the puffer (*Spheroides maculatus*) at 19.5°C. no convulsions were observed (Gray, 1928).

Gray suggests that metabolic rate may be more important than temperature in determining the time required for the production of convulsions by insulin in fish, since the trout at 4°C. (convulsions in 24–60 hrs.) may have as high a metabolic rate as the catfish at 21°C. (convulsions in 51–53 hrs.). Huxley and Fulton suggested that the action of insulin is not itself altered by change in temperature, but that "its speed of action is dependent upon the metabolic rate of the animal itself." Since, for poikilotherms, the metabolic rate increases with increase in temperature (Krogh, 1941), it is difficult in this case to differentiate the effects of metabolic rate and of temperature upon insulin action. In

view of the effect of temperature upon the rates of biological processes, it is quite possible that the action of insulin itself as well as the rate of metabolism is directly influenced in a similar manner by temperature.

Homeotherms. The action of insulin in mammals is influenced by environmental temperature. Temperatures below about 30°C. increase the metabolic rate of homeotherms. Mortality is higher and convulsions appear more rapidly in rats and mice after administration of insulin if the air temperature is maintained around 30°C. than if it is lower (Voegtlin and Dunn, 1923; Hemmingsen and Krogh, 1926; Hemmingsen, 1939). The CD_{50} in mice is about 80 times as great at an environmental temperature of 20°C. as at 40°C. (Chen et al., 1943b). Rabbits, fasted for 18 hours at 33°C., injected with insulin and exposed for 7 hours at the same temperature, developed convulsions (Johlin, 1944). With the same dose of insulin and both exposure temperatures at 20°C., convulsions again appeared. However, when the fasting period was at 33°C. and the experimental period at 20°C., convulsions did not appear. Blood sugar level was the same in all animals. The author suggests that hyperventilation at 33°C. produced a temporary uncompensated acidosis at 20°C. which restrained the convulsions. An inhibiting effect of high atmospheric pCO_2 on insulin convulsions has been reported (McQuarrie et al., 1940).

The action of insulin on homeotherms has also been investigated during hypothermia. Cassidy, Dworkin and Finney (1925a) found that in dogs and cats (fasted 24 hrs. and anesthetized with amytal) insulin lowered the blood sugar with approximately the same rapidity at body temperatures of 38° and 25°C. In some animals at 25°C., however, there was a delay of 40–60 minutes before the fall in blood sugar commenced, but after that time the form of the blood sugar curve did not differ appreciably from that at 38°C.

Krogh (cf. Macleod, 1924) observed that at room temperature insulin rarely produced convulsions in mice, but only a comatose condition and a fall in body temperature, but that if the animals were kept at an environmental temperature of 28°C. (and a fall in body temperature prevented), convulsions of the usual type were seen. In dogs and cats with blood sugar below 45 mgm. per cent, insulin convulsions did not occur at rectal temperatures of 25°C., even though in two dogs blood sugars as low as 16 and 36 mgm. per cent were found. Convulsions did occur when the animals were re-warmed to 38°C. (Cassidy, Dworkin and Finney, 1925-b). Lowering of the blood sugar by means of insulin abolished shivering in hypothermic cats and dogs; shivering could be restored by means of glucose (Cassidy et al., 1925-b). Fowls in which the blood sugar was reduced to about 40 mgm. per cent by means of insulin did not at once shiver upon immersion in water at 20°C. (which produces shivering in the normal hen), but shivering began coincidentally with a rise in blood sugar (Cassidy, Dworkin and Finney, 1926). Woodchucks (*Arctomys monax*), given an amount of insulin sufficient to produce marked hypoglycemia, lose the power of temperature regulation, and hypothermia occurs when they are placed in a cold environment (Dworkin and Finney, 1927).

Tyler (1939) found that the body temperature of rabbits could be reduced to

about 28°C. in 3 hours by the administration of 3 U./kgm. insulin, wetting the animals with alcohol, and placing them in front of a fan. If animals were first cooled to 25–28°C., then given 0.5 U./kgm. insulin I.V., the rate of fall of blood sugar was about the same as in animals at normal body temperature, but 6–10 hours were required for it to return to normal in the cooled animals as compared to 3–5 hours in uncooled controls. This prolonged hypoglycemia may be due to decreased glyconeogenesis at low temperature or to a prolongation of insulin action. Survival after massive doses of insulin was prolonged at body temperatures of 25°–30°C. The hypothesis was offered that both the increase in the survival period and the inhibition of convulsions in cooled animals receiving insulin are the result of the decreased metabolic rate and the subsequent decreased demand for substrates by the vital centers of the brain (Tyler, 1939). Brain damage occurred earlier in cats rendered hypoglycemic by insulin when the body temperature was high than when very marked falls occurred. This was investigated more completely in cats which were maintained at body temperatures of 31°–34°C. or 36°–39°C. (Tyler, 1940). The extent of brain damage (determined histologically) was greater in those at 36°–39°C. for a given duration of hypoglycemia than in those at 31°–34°C. When the body temperature was maintained at normal levels it was considered that a greater relative degree of tissue anoxia resulted than when the temperature was permitted to fall.

Since the mechanism of production of insulin convulsions is not definitely known (cf. Jensen, 1938, p. 157), the reason for prevention of convulsions by hypothermia is not clear. It seems probable that convulsions occur at blood glucose concentrations of essentially zero (Dotti and Hrubetz, 1936) and that they thus depend upon failure of carbohydrate supply to the brain (Gerard, 1938). At low temperatures, when brain metabolism is greatly reduced, convulsions may be prevented by substrate concentrations inadequate to prevent them at normal body temperatures.

Thyroid. Unfortunately the effect of thyroxin or thyroid administration has not been thoroughly studied in homeotherms at body temperatures other than normal. O'Connor (1936) reported that thyroid increased the oxygen consumption of anesthetized, curarized rabbits about 50 per cent at all body temperatures from 22° to 39°C. Other workers have reported chiefly the results of experiments at varied environmental temperature, which has a marked effect upon the survival time of mammals treated with thyroxin or desiccated thyroid. Stoland and Kinney (1919) reported that rats given 0.2 gram of thyroid daily lived for 7.3 days at an environmental temperature of 32°C., for 22 days at 25°C. and for 32 days at 18°C. At 33°C. environmental temperature rabbits lose weight when given 50–75 mgm. per kgm. desiccated thyroid daily, while at 1°C. the body weight is maintained (Draize and Tatum, 1932). More complete data of a similar nature are given by Bodansky, Pilcher and Duff (1926), who found that the period of survival of rats given 2 mgm. thyroxin daily increases with a decrease in environmental temperature. Dempsey and Astwood (1943) found, using rats treated with thiouracil, that daily doses of 9.5 micrograms, 5.2 micrograms and 1.7 micrograms of thyroxin daily were required to maintain the weight of the thy-

roid gland at environmental temperatures of 1°, 25° and 35°C. respectively. Guinea pigs, given 0.3 mgm. thyroxin daily, showed a greater increase in rectal temperature than did untreated animals when placed at a high environmental temperature. At the high temperature CO_2 production increased in the thyroxin-treated animals and decreased in normal animals (Okamura, 1939).

In contrast to the marked effects of thyroid or thyroxin in increasing the metabolic rate of homeotherms, the metabolic rate of frogs and small fishes does not appear to be increased by the administration of either substance (Drexler and v. Issekutz, 1935; Henschel and Steuber, 1931). Tadpoles of *R. temporaria* exposed to thyroid in solution and then placed at environmental temperatures of from 3° to 30°C. undergo complete metamorphosis only at temperatures above 5°C. (Huxley, 1929). Below 5°C. metamorphosis is not complete, even when the animals are later removed to room temperature. The implications of this finding are discussed by Needham (1942).

MISCELLANEOUS DRUGS. *Acetylcholine.* Clark (1926) reported that temperature produced no certain effect upon the amount of action of acetylcholine on isolated frog heart or rectus abdominis. An increase in temperature, however, increased both the rate of action and the rate of washing out. Laubender and Kolb (1936) obtained similar results on the isolated frog rectus abdominis.

The slowing of the heart produced by intravenous administration of acetylcholine in dogs was much greater at 25°C. body temperature than at 36°C. (Tournade et al., 1938; Grosse-Brockhoff and Schoedel, 1943c). Arrhythmias frequently occurred at the lower temperature with doses which did not disturb the cardiac rhythm at 36°C. Vagal stimulation, at an intensity ineffective at normal temperature, produced marked effects at 25°C. These changes were suggested as being due to a decreased activity of choline esterase at low temperature. Acetylcholine, whether injected or formed as the result of vagal stimulation, would thus be present in higher concentration and for a longer period of time at 25°C. than at 36°C.

Dicumarol. The influence of fever upon the action of dicumarol, as measured by prothrombin time, has been studied by Richards (1943). Prothrombin time, determined 24 hours after the administration of dicumarol, was five times as long in rats with a mean body temperature of 39.6°C. as in rats with a mean body temperature of 37.3°C. These data are in accord with the view that reduction in plasma prothrombin level is dependent upon a chemical or enzymatic reaction involving dicumarol.

Carbon dioxide. Frogs were not anesthetized with 25 per cent CO_2 during several days' exposure, and were anesthetized only after several hours with 50 per cent CO_2 (Winterstein, 1919). Narcosis in rats and dogs was produced by 11 per cent CO_2 at an environmental temperature of 5°C. in 3 to 6 hours (Barbour and Seevers, 1943). At room temperature such CO_2 concentration does not result in narcosis. Body temperature of the narcotized animals fell and it was necessary to raise the environmental temperature after induction of narcosis in order to prevent death from hypothermia. Barbour and Seevers suggest that a sudden increase in tissue CO_2 tension decreases total oxidative metabolism and reduces

activity of the tissues, including the central nervous system. At 5°C. decrease in total heat production results in lowering of body temperature to the point of narcosis.

Oxygen. The effects of oxygen at increased pressure have recently been reviewed by Bean (1945). Poikilotherms appear to be more resistant to increased oxygen pressure than are homeotherms. The toxic action of oxygen at high pressure is enhanced by increase in environmental temperature. (References and details are given by Bean.)

Tetanus toxin. This toxin is reported to be relatively inactive in frogs at room temperatue and below, but to become toxic upon warming the animals (Courmont and Doyon, 1893).

Abrin. Abrin was reported by Lesné and Dreyfus (1908) to be several hundred times more toxic in frogs at 29°C. than at room temperature. Guinea pigs maintained at an environmental temperature of 39°C. were killed by a dose which was not fatal in animals at lower temperatures.

Atoxyl. Atoxyl (sodium arsanilate) was found to be much less toxic in frogs than in mammals (Muto, 1910). Sanno (1911) determined the lethal dose in frogs at 8°C. to be about 15 grams per kgm. and at 37°C. to be about 1.3 grams per kgm. These results may be explained by the conversion of sodium arsanilate by the tissues to a more toxic reduction product, a reaction which is more rapid at higher temperatures.

Nicotine. Hill (1909) determined the Q_{10} of the action of nicotine on isolated frog muscle to be 2.8 between 7° and 17°C. Laubender and Kolb (1936) obtained a Q_{10} of 2.2. This, together with concentration-action data, was taken to indicate a chemical mode of action, but Clark (1937) points out that there is no guarantee "that at different temperatures fixation of equal quantities of drug will produce an equal effect."

In mice cooled to a body temperature of 20°C., nicotine is more toxic than in control animals at normal body temperature (Leser et al., 1940).

Procaine and cocaine. Procaine, injected intramuscularly, was found to be most toxic in mice during the summer months (Sievers and McIntyre, 1937). The effects of environmental temperature were investigated more completely by determining the fatal dose in mice exposed to a given temperature ranging from about −10°C. to 40°C. for two hours before injection. The largest minimum lethal doses were found in the temperatue range 7 to 24°C., with decrease in MLD at temperatures both below and above this range. Below 7°C. the body temperature of the mice was probably reduced by the preliminary exposure period.

In mice cooled to 20°C. body temperature, both procaine and cocaine were more toxic than in control animals, and the survival time was prolonged in the cooled animals (Leser et al., 1940).

Discussion. The complexity of the effects of temperature upon the action of drugs is at once apparent from the foregoing consideration. It is however, possible and highly desirable to classify the available data in such a way that general conclusions may be drawn. It is no longer possible to accept without

reservation the conclusion of Richet (1889, p. 214): "les actions toxiques, étant des actions chimiques, varient avec le température de l'animal. Elles sont d'autant plus actives que la température de l'animal est plus élevée." As Bělehrádek (1935, p. 90) clearly points out: "In the action of various chemicals upon living cells and organisms under varied temperature, two separate phenomena must be clearly distinguished, viz., (a) the effect of temperature upon the rate at which a given substance permeates into the system or at which it produces definite changes in the system, and (b) the variations of the effective concentration, or of the final concentration, or of the mode of action, of such a substance, with temperature."

Rate of action. When pharmacologically active agents are administered to intact animals at different levels of body temperature, the rate at which they act is usually measured by the elapsed time between administration and the occurrence of a response. This latent period is determined (1) by the time required for the drug to reach the site of action in sufficient concentration to produce an effect, and (2) by the time required for the interaction between the drug and the tissues.

The time between administration and the appearance of effect is very greatly influenced by the route of administration. Following intravenous administration, the effect of temperature upon this phase of action is a result of the effect of temperature upon the circulation time. In man, during general hypothermia, the circulation time is found to be two to three times as long as at normal body temperature (Oppenheimer and McCravey, 1941). Following subcutaneous or intramuscular administration, it is to be expected that rate of absorption will be dependent upon blood flow in the region of injection. Local cooling, operating through local reduction of blood flow, is an effective means of delaying absorption. Part of the effect of low temperature in decreasing the toxicity of digitalis in frogs, when the drug is administered via the lymph sac, is the result of the slower absorption of digitalis at low temperature (Sollmann et al., 1915).

The effect of temperature upon the rate of interaction between the drug and the tissues of the animal is often obscured by the effect of temperature upon the rate of absorption. This interaction is believed to be chemical in many instances. It is therefore to be expected that the reaction will be temperature-sensitive, and that the rate at a given temperature will be in general about two to three times the rate at a temperature 10°C. lower (Q_{10} = 2–3). Experimental data describing the effect of temperature upon the rate of interaction of drugs and tissues have provided temperature coefficients of about this magnitude (Clark 1937; Bělehrádek, 1935). Although such temperature coefficients indicate that the actions produced are chemical in nature, it must be remembered that changes in temperature produce alterations in the tissues themselves: changes in rate of nerve conduction, metabolic rate, viscosity, etc. (cf. Clark, 1937; Lucas, 1908; Samojloff, 1925). There may still be doubt, in spite of such temperature coefficients, as to whether the process is physical or chemical.

Intensity and duration of action. Intensity of drug action is dependent upon the concentration of the drug obtaining at the site of action, and upon physiolog-

ical factors which may alter the response of the tissue acted upon. The concentration of drug attained in a tissue is the resultant of the processes of absorption, excretion and detoxification. All of these processes may be influenced by temperature, and each may be altered to a different degree. The rate at which an inactive form of drug is changed into an active form may also be altered by temperature.

The duration of action of a drug is determined by the length of time during which a concentration above the threshold level is maintained at the site of action. This is influenced both by the rate of absorption or of activation and the rate of excretion or destruction. The influence of temperature upon the rate of absorption has already been mentioned. Change in body temperature also has a profound effect upon the rate at which a drug is destroyed by the tissues. Although these detoxification processes are in most cases not yet well enough understood to describe complete systems, it is clear that for a number of drugs (acetanilide, histamine, atropine, epinephrine, alcohol) these reactions are enzymatic in nature (Bernheim, 1942). The short-acting barbiturates are probably also detoxified enzymatically. Thus it must be true, in view of the very well established effect of temperature upon rate of enzyme action (Sizer, 1943), that decrease in body temperature will slow the enzymatic detoxification of these substances; the Q_{10} for such a process should lie in the range 2 to 4. Experimental evidence that the duration of action of acetylcholine, caffeine, epinephrine, alcohol and pentobarbital are markedly increased at low body temperature levels has been provided. In contrast, the action of barbital (which is largely excreted in the urine) is not prolonged during hypothermia. It is probable that other examples of this type of temperature effect will be found.

Colchicine and atoxyl are changed by the tissues to more active products and thus, by reason of slower activation, are less toxic at low body temperatures.

The effect of body temperature upon the rate of excretion of drugs by the kidney does not appear to have been investigated. It is reported that kidney function is maintained during hypothermia in man (Smith, 1942), so that large alterations in urinary excretion by changes in body temperature may not be expected to occur.

Changes in lethal dose with changes in temperature may result from a greater effect of temperature upon either rate of absorption or rate of elimination. Variations in lethal dose produced by changes in body temperature are complicated at both extremes by the effect of temperature upon the organism. For example, during hypothermia the summated depressant effects of cold and of anesthetics may lead to respiratory failure with lower doses of anesthetic than are lethal at normal body temperature.

When the contributions of experimental investigation of the effect of temperature upon drug action carried out during the last 60 years are considered, there is even more reason today than there was in 1885 to agree with Brunton that "All these things show that the *definition* of the *action of a drug* . . . must be still further modified, and we must define it as the reaction between the drug and the various parts of the body at a *certain temperature*."

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LIPINS AND LIPIDOSES

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RECENT ADVANCES IN THE CHEMISTRY AND PATHOLOGY OF THE LIPINS (PHOSPHATIDES AND CEREBROSIDES). The pathological chemistry of the phosphatides and cerebrosides is so far chiefly based on observations involving the "chemical anatomy" of these substances rather than their functions in the cells. The distribution of these substances in the animal tissues is of great interest for clinical pathology since it has been found that, during certain diseases, specific phospholipids or cerebrosides accumulate in large amounts in organs such as brain, liver, lung, and spleen. In such cases the accumulations of specific lipins are so characteristic that frequently the correct diagnosis is possible only after the chemical identification of the lipins in certain tissues.

I. CHEMISTRY OF THE LIPINS. The customary classification of the lipins into the groups of monoaminophosphatides, (lecithins and cephalins), diaminophosphatides (sphingomyelins), and cerebrosides has to be modified in order to incorporate all lipins known at the present time. This classification originated at a period in which it was believed that the various members of each group differed from one another only in the nature of their component fatty acids. This assumption, however, holds only for the lecithins, whereas each one of the other groups includes individuals which differ also in the structures of their non-fatty acid components from other individuals of the same group. Furthermore, some phosphatides of the acid-fast bacteria do not fit into any group of the usual classification.

The following scheme contains the lipins known at the present time:

1) *Monoaminophosphatides:* Fatty acid esters of a phosphorylated polyvalent alcohol, combined with a nitrogen-containing group. Their ratio P:N is 1:1. (Some monoaminophosphatides contain other organic groups in addition to those already mentioned.)
   - A. *Lecithins.* Phosphoric acid diesters of diglycerides and choline.
     Lysolecithins. Phosphoric acid diesters of saturated monoglycerides and choline.
   - B. *Cephalins.* All known cephalins contain their total nitrogen in form of a primary amino group (ethanolamine or serine).
     - a.) Phosphatidyl ethanolamines.
       Phosphoric acid diesters of diglycerides and ethanolamine.
     - b.) Phosphatidyl serines.
       Hydrolysis products: Fatty acids, phosphoric acid, polyvalent alcohols, serine.
2) *Plasmalogens* (*Acetalphosphatides*): Phosphoric acid diesters of a higher fatty aldehyde acetal of glycerol and of ethanolamine.
3) Inositol-phosphatides obtained from brain, soybeans and bacteria. See text and supplement regarding their composition.

4) *Phosphatidic acids, cardiolipins:* Hydrolysis products: Fatty acids, polyvalent alcohols, phosphoric acids (bound as a monoester).
5) *Phosphatides of acid-fast bacteria:* Hydrolysis products: Phosphoric acid, polyhydroxy compounds (such as carbohydrates, inositol), fatty acids with straight and branched chains.
6) *Diaminophosphatides (Sphingomyelins):* Acid amides of sphingosine with fatty acids (ceramides) in ester linkage with phosphorylcholine.
7) *Cerebrosides:* Acid amides of fatty acids with sphingosine or dihydrosphingosine in glucosidic linkage with galactose or glucose.
8) *Gangliosides:* Structure unknown; hydrolysis products: sphingosine, neuraminic acid, fatty acids, and galactose or glucose.

Fatty acids in lipins. Since the fractionation of lipins is possible so far only to a limited extent, the analysis of the fatty acid components obtained by hydrolysis of the lipid fractions is at present the only method which permits an insight into the finer composition of the phospholipids isolated from tissues. Because of the probability of a metabolic relationship between the various lipins and the neutral fats, a comparison between the structures of their component fatty acids is of physiological interest in regard to the intermediary metabolism of lipids. Comparative studies of the fatty acids occurring in the lipins of various organs are all the more valuable as our knowledge of the enzymes involved in the transformation of fatty acids is as yet too scarce to permit another experimental approach to the intermediary metabolism of the lipids. Systematic investigations of the fatty acid components of phospholipids have been carried out in the laboratories of Anderson (1), Bloor (2), Hilditch (3), Klenk (4, 5), Thannhauser (6). Considering the large number of physiological fatty acids, the complicated possibilities of their isomerism and the technical difficulties of their fractionation, it is obvious that the available data, numerous as they are, permit only relatively few conclusions of a more general physiological interest. For this reason the important investigations in this field can be discussed in this article only to a very limited extent.

Some recent advances in the methods of separation and identification of fatty acids. The fractional distillation of fatty acids or their methyl esters under highly reduced pressure has been successfully applied to the separation of small amounts of fatty acid mixtures. Special distilling columns for this purpose have been described by several authors (7, 8, 9, 10).

Brown and his co-workers (11) developed the fractional crystallization of fatty acids or their methyl esters from organic solvents at very low temperature. This technique is of particular importance for the isolation of unsaturated fatty acids such as linoleic, linolenic, and arachidic acid because it avoids the formation of conjugated double bonds caused by the usual methods of purification.

Spectrophotometry in the ultraviolet range has been successfully applied to estimate the percentage of conjugated double bonds in mixtures of unsaturated fatty acids (12).

Some data concerning the infra red spectrum of fatty acids are compiled in the review published in the Analytical Edition of Industrial and Engineering

Chemistry (13). Infra red spectroscopy, however, has not yet been applied to tissue analysis.

Analyses of the X-ray diffraction pattern have yielded valuable results concerning the structure of natural higher fats (14, 15).

The investigation of monolayers of fatty acids has led to important conclusions regarding the structure of fatty acids with branched chains (16).

Fatty acids in *animal* lipins: Klenk (17) concludes from the results of extensive investigations embracing species of all classes of vertebrates that the ether-soluble phosphatides contain fatty acids with even carbon chains of 16 to 22 C atoms whereas acids with 24 C atoms are exclusively found as components of cerebrosides and sphingomyelins. The fatty acid mixture obtained from ether-soluble lipins consists of individuals of various degrees of unsaturation. On the average the number of double bonds per fatty acid molecule of the monophosphatide fraction increases with the number of C atoms. The composition of the fatty acid mixture obtained from the neutral fat of liver is remarkably similar to that of the monoaminophosphatides. The acids of the storage fats, however, differ strongly from those of the monophosphatides inasmuch as the highly unsaturated fatty acids with 20 and 22 C atoms are absent. The similarity between monoaminophosphatides and liver triglycerides on the one side and the differences between phosphatides and storage triglycerides on the other side in respect to their component fat acids is interpreted by Klenk as a consequence of metabolic relations between phosphatides and triglycerides in the liver. Klenk assumes that the liver is the organ in which the highly unsaturated fat acids are incorporated into the phospholipids. A metabolic relationship between fat acids of various chain lengths has become even more plausible since the recent elucidation of the structure of arachidonic acid (18, 19, 20). The formula of arachidonic acid is that of a 5, 8, 11, 14 eicosatetrenoic acid. It is interesting to note that the location of the double bonds in the chains of the terminal C atoms of arachidonic and linoleic acids is identical. Dolby, Nunn, and Smedley-MacLean (18) discuss the possibility that linoleic acid would represent the physiological precursor of arachidonic acid. Arachidonic acid could be formed from linoleic acid by condensation of the carboxyl group with a two carbon chain and subsequent desaturation.

Arachidonic acid:

$$CH_3(CH_2)_4CH{:}CHCH_2CH{:}CHCH_2CH{:}CHCH_2CH{:}CHCH_2CH_2CH_2COOH$$

Linoleic acid:

$$CH_3(CH_2)_4CH{:}CHCH_2CH{:}CHCH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$$

It can be gathered from the preceding discussion that the differences between the fatty acid composition of phosphatides, liver fats, and body fats involve mainly the fraction of the highly unsaturated fat acids. This is of particular interest inasmuch as Burr (21) discovered some time ago that some of these acids, namely, linoleic and arachidic respectively are "essential fatty acids."

It was emphasized in the introduction to this chapter that, as a rule, it is not yet possible to separate monoaminophosphatides which differ from each other exclusively in regard to their component fat acids. Since the purified monophosphatide fractions usually contain saturated and unsaturated acids in approximately equal amounts, it was frequently assumed that the natural monophosphatides contain in each molecule one saturated and one unsaturated fat acid.

Lesuk and Anderson (22), however, succeeded recently in isolating a saturated lecithin, dipalmitolecithin, from the ether-insoluble lipid fraction of larvae of cysticercus fasciolaris.[1] The insolubility of this natural saturated lecithin in ether is in accordance with the properties of other saturated lecithins obtained by hydrogenation of natural lecithins and of synthetic saturated lecithins (23). Thannhauser and his co-workers (24) encountered saturated monoaminophosphatides in the fraction of the ether-insoluble phospholipids obtained from animal tissues such as brain and lung. In view of these findings it is obvious that the classical fractionation of the lipins into an ether-soluble and ether-insoluble part can no longer be interpreted as a separation of mono and diaminophosphatides.[2]

Fatty acids of cerebrosides and sphingomyelins. The component fatty acids of the cerebrosides and sphingomyelins differ from those of the monoaminophosphatides. So far, it seems that the latter group of animal and plant phosphatides contains only fatty acids with even numbers of C atoms up to 22. The majority of sphingomyelins and cerebrosides, however, are characterized by component fatty acids with chains of 24 and more C atoms, namely, lignoceric acid, n-tetracosanic acid, $C_{24}H_{48}O_2$ m.p. 83–84°; cerebronic acid, α-hydroxylignoceric acid, m.p. 100–101°, α_D^{20} (Pyridine) + 3.41°; nervonic acid, 9,10 dehydrolignoceric acid $C_{24}H_{46}O_2$ m.p. 40–41°; α-hydroxynervonic acid $C_{24}H_{46}O_3$ m.p. 65°; α_D^{20} (Pyridine) + 2.87. The structure of cerebronic acid which was the object of discussion for many years now appears to be definitely established by its synthesis (26).

Until very recently the chain length of the fatty acids isolated as hydrolysis products of these lipins did not seem to exceed 24 C atoms. Klenk and Schumann(27), however, succeeded in isolating from the hydrolysis products of a cerebroside fraction a hexacosanoic acid which crystallized from acetone in bright scales and melted at 45–45.5°.

Fatty acids in phosphatides of acid-fast bacteria. Anderson and his co-workers (1) demonstrated in a series of investigations the presence of saturated fat acids with branched chains in the phosphatides and in the acetone-soluble lipids of acid-fast bacilli. These substances have lower melting points than their isomers with straight chains and appear usually as liquids at room temperature.

Tuberculostearic acid (28, 15), 10-methyl stearic acid, $C_{19}H_{38}O_2$, has been obtained in pure form from the phosphatides and the acetone-soluble lipids of

[1] The analysis of this particular tapeworm was undertaken because its larvae produce a sarcomatous growth in the surrounding tissue of the host.

[2] The ether fractionation of phospholipids has recently been criticized by Sinclair (25) for other reasons.

tubercle bacilli and melts at 10–11°C. Its optical inactivity is attributed by Spielman (28) to the remoteness of the asymmetric C atom from the carboxyl group rather than to racemisation. Synthetic dl-10-methyl stearic acid melts at 20–21°. The difference in the melting points between natural tuberculostearic acid and synthetic dl-10-methylstearic acid is not yet definitely explained. It is possible that the natural compound differs from the synthetic only in its stereoisomeric properties. This view is supported by the results obtained with comparative analyses of the x-ray diffraction pattern of both compounds (15). Natural tuberculostearic acid produces no specific biological reactions after injection into animals.

Phthioic acid, $C_{26}H_{52}O_2$, was discovered by Anderson and Chargaff (29, 30) in the phosphatides and acetone-soluble lipids of all strains of tubercle bacilli. It is dextrorotatory ($\alpha_D^{20°} = +12.6°$) and doubtlessly represents a fatty acid with branched chains although its structure is not yet known in detail. Stenhagen and Ställberg (31) conclude from their studies on monolayers of phthioic acid that its structure should be expressed as that of a trisubstituted acetic acid with two long and one short chain. Phthioic acid as well as the phthioic acid-containing phosphatide fractions of tubercle bacilli are of great biological interest since these substances produce typical tubercular changes after injection into tissues (32).

Phytomonic acid (33), $C_{20}H_{40}O_2$, m.p. 24°, has been isolated from the phosphatides and the acetone-soluble lipids of the crown gall bacillus (phytomonas tumefaciens). The low melting point suggests the assumption of a branched chain structure. The discovery of a branched chain fatty acid in this micro-organism demonstrates the fact that this type of fatty acid is not limited to acid-fast bacteria.

Mycocerosic acid, $C_{30}H_{60}O_2$, m.p. 27–28°, was obtained by Ginger and Anderson (34) from the wax present in the cell residues from the preparation of tuberculin. It forms an ether-soluble lead salt and is levorotatory ($\alpha_D^{20°}$ Chloroform $= -5°$ to 6°).

No fatty acids with branched chains have been found in yeast phosphatides (35) and in tuberculous lung tissue (36).

Recently Weitkamp (37) reported observations suggesting the presence of branched chain fatty acids in wool fat (degras). Weitkamp's conclusions have been confirmed by Velick and English (38) who synthesized d-14-methyl palmitic acid and found it identical with the natural acid obtained from wool.

Another group of acids which are very characteristic components of the acid-fast bacteria, the *mycolic acids*, (39) are apparently *not* present in the phosphatides of these micro-organisms. These substances have been discovered by Anderson and his co-workers as constituents of the so-called waxes of the acid-fast bacteria and are hydroxyfatty acids with branched chains of very high molecular weights. Mycolic acids account for a large part of the lipids of acid-fast bacteria. They are solid at room temperature and soluble in ether. On pyrolysis (heating at 250° to 300° under highly reduced pressure) they decompose yielding fat acids of straight or branched chains. For example the mycolic acid obtained from the wax of human tubercle bacteria (40) has the

probable formula $C_{88}H_{176}O_4$, melts at 54–56°C, and is dextrorotatory ($\alpha_D^{20°} = +1.8°$). The noncarboxylic oxygen atoms are present in the form of one hydroxyl and one methoxyl group. On pyrolysis mycolic acid yields n-hexacosanic acid.

The mycolic acids are of great biological significance being probably responsible for the acid fastness of the mycobacteria.

Glycerophosphate in phosphatides. Theoretically, all phosphatides containing glycerophosphate could be derivatives either of alpha or of beta glycerophosphate. No method permits at present the separation or differentiation of these two possible series of phosphatides. Some earlier authors attempted to obtain information concerning this structural problem by investigating the mixture of the two glycerophosphates obtained after hydrolysis of natural phosphatides. However, Folch (41) was able to demonstrate that the structure of glycerophosphate obtained by acid or alkali hydrolysis of phosphatides does not permit conclusions as to the structure of the intact phosphatide molecule since the phosphate linkage in glycerophosphate undergoes rearrangements on boiling with acids or alkalis. Thus it appears that the solution of this structural problem has to await the development of new methods for the splitting of phosphatides, perhaps the use of hydrolyzing enzymes.

Lecithins. The method for the preparation of lecithin (Levene and Rolf, 42) has been simplified by Pangborn (43).

A new lecithin, dipalmitolecithin, has been isolated by Lesuk and Anderson (22) from the larvae of cysticercus fasciolaris. With this discovery, a saturated lecithin has been found for the first time in biological material. Some properties of this lecithin have already been discussed (p. 278).

Aminoethanol cephalin, phosphatidylethanolamine. This fraction which until recently was considered to be the only constituent of cephalin, has now been obtained reasonably free of other phosphatides by Folch (44). Its components appear to be exclusively fatty acids, glycerol, phosphoric acid, and ethanolamine. In some important properties it differs considerably from the "cephalin" described by earlier investigators. Phosphatidylethanolamine from ox brain is easily soluble in alcohol and contains only traces of ash. It represents a slightly sticky white powder which, on standing in the dark in an evacuated desiccator for two weeks, acquires a tan color which later turns into a deep brown. Its iodine number of 78 suggests the presence of two double bonds for each atom of P.

Phosphatidyl serin was obtained by Folch (44) from the crude cephalin fraction of ox brain by fractionated precipitation of cephalin solutions in chloroform with alcohol. Its components are fatty acids, glycerol, phosphoric acid, and l(+)-serin. When the substance is prepared from brain cephalin without acid treatment, it contains approximately 12 per cent of ash, which appears to originate from the salts of the serine phosphatide with potassium, sodium, and calcium. The iodine number of 33 would indicate the presence of one double bond per atom of phosphorus. According to the description of Folch, phosphatidyl serine seems to be soluble in alcohol to a considerable extent.

Chargaff, Ziff and Rittenberg (45) found that amino acid-containing phosphatides are present in the phosphatide fractions of various animal organs such as brain, lung, heart, and liver. In brain phosphatides the amino acid nitrogen accounts for approximately 30 per cent of the amino nitrogen, in lung for 23 per cent. It is interesting that egg yolk phosphatides are practically free of amino acid nitrogen.

Inositol phosphatides, lipositol. The presence of inositol in phosphatides was first demonstrated in Anderson's laboratory (46, 47) in 1930 in bovine tubercle bacilli; in 1939, Klenk and Sakai (48) reported the isolation of inositol monophosphate from soy bean cephalin after acid hydrolysis; in 1942, Folch and Woolley (49) found inositol in the least alcohol-soluble fraction of brain cephalin. According to these authors, the inositol-containing fraction represents approximately one-fourth of the total brain cephalin.

By fractionated precipitations of brain cephalin Folch succeeded in arriving at products containing 6.8 per cent inositol. These fractions still contained serine as well as glycerol and have certainly to be considered as mixtures.* Woolley (50) prepared from soy bean phosphatides a cephalin fraction with 16 per cent inositol; this fraction which he called lipositol is apparently free of glycerol and serine. The hydrolysis of lipositol with acids respectively alkali yielded in equimolecular proportions phosphoric acid, inositol, galactose, tartaric acid, ethanolamine, oleic acid, and a mixture of saturated fatty acids (palmitic, stearic, and cerebronic acid) the sum of which was equimolecular to the amount of oleic acid. The phosphoric acid appears to be esterified with inositol which is probably linked to the carbohydrate as galactoside in the lipositol molecule. Woolley reported in his paper on soy bean lipositol that inositol phosphatide fractions of brain contained likewise galactose and tartaric acid (50, p. 583).

Soy bean lipositol is only slightly soluble in methanol, ethanol, dioxane, glacial acetic acid. It is insoluble in dry petroleum ether, dry benzene, dry ether, or dry chloroform, but soluble in the moist solvents The preparation contains approximately 12 per cent of ash, which is so firmly bound that it cannot be removed by dialysis of a lipositol suspension in hydrochloric acid.

No other nitrogen-containing group, besides colamine, has been found among the hydrolysis products of soy bean lipositol. In particular amino acid nitrogen was found to be absent. Alkali hydrolysis yielded considerable amounts of colamine, while only very small amounts of the base were obtained after acid hydrolysis.

Acetal phosphatides; plasmalogens. This group of phosphatides was discovered by Feulgen during histochemical studies on the staining of tissues by fuchsin sulfurous acid. This reagent which produces a purple color with aldehydes permits, after treatment of the cells with sulfuric acid, a selective staining of the chromatin structures of the nuclei due to the aldehyde groups appearing on hydrolysis of thymonucleic acid. Feulgen introduced the term "nucleal" for his staining method. He observed very soon that the staining effect of

*See supplement.

the nucleal reagent was not strictly limited to the cell nucleus, but included in many tissues the cytoplasm, although with a lesser intensity. The staining of the cytoplasm was successful only in undefatted slices while that of the nuclei was not influenced by any treatment of the tissues with lipid solvents. Feulgen concluded that different substances were responsible for the staining of nuclei and cytoplasma; he proposed, therefore, for the unknown carriers of the fuchsin sulfurous acid staining in the cytoplasm the term "plasmal". Since he had already found in his earliest histochemical experiments that the staining of the plasma was strongly accelerated and intensified by preliminary treatment of the tissue with acid or with mercuric chloride he postulated that the plasmal was not present in the cell as such, but in the form of a hypothetical precursor which he designated as plasmalogen.

Attempts to isolate plasmal and plasmalogen resulted in the observation that these substances were found exclusively in the phospholipid fractions of the investigated tissues. On addition of a small amount of mercuric chloride to phospholipid suspensions from brain, muscle, or heart, a strong plasmal reaction was immediately obtained. Feulgen, Imhäuser, and Behrens (51) were able to isolate the aldehyde from horsemuscle phosphatides by steam distillation and subsequent condensation with semicarbazone as a crystallized semicarbazide and to demonstrate that this substance consisted chiefly of palmitic aldehyde semicarbazide contaminated with a small amount of stearic aldehyde semicarbazide. An improved method for the preparation of plasmal semicarbazide from horsemeat has been reported by Behrens (52). He was able to isolate 1–1.5 gram of plasmal semicarbazide from 10 kgm. horsemeat. Recently Anchel and Waelsch (53) isolated fatty acid aldehydes from relatively small amounts of tissues. The proportion of palmitic and stearic aldehydes differs in plasmals from different organs and species. It is certain that all plasmals contain besides palmitic and stearic aldehyde at least one other fatty aldehyde which so far could not be isolated on account of the solubility of its semi-carbazide.

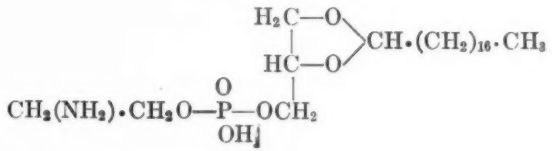
The isolation of plasmalogen was accomplished by Feulgen and Bersin (54) on the basis of its relative stability against alkali. When the phosphatide emulsion from beef muscle was treated with sodium hydroxide, a considerable part of the plasmalogen remained intact while the contaminating phosphatides were saponified. From the reaction mixture, plasmalogen could be precipitated together with the fatty acids by converting them into the brucine salts. After extraction of the brucine soaps with acetone, the plasmalogen could be isolated by repeated treatment of the acetone-insoluble residue with benzene in which dry pure plasmalogen is not soluble. The substance is then crystallized from alcohol at room temperature.

Hydrolysis products of plasmalogen. By treatment with mercuric chloride, plasmalogen is split into a higher aldehyde and colamine glycerophosphate (this substance crystallizes in fine needles from alcohol and is very soluble in water, but insoluble in cold alcohol, m.p. 86–87°). When heated with 3.3 per cent sodium hydroxide for 6 hours, plasmalogen is split into colamine and plasmalogenic acid which can be isolated as lithium salt. Feulgen and Bersin

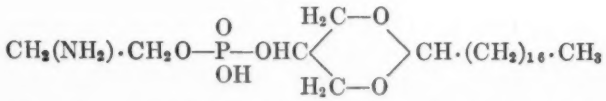
were able to demonstrate that this split-product is composed of glycerophosphate and a fatty aldehyde in equivalent amounts.

Structure of plasmalogen. Feulgen and Bersin concluded from their observations that plasmalogens are acetals of fatty aldehydes with colamine glycerophosphate. The structure of stearal plasmalogen would be represented by either of the following formulae:

(1)



(2)



The assumption of an acetal linkage is supported by the stability of the nitrogen-free group against alkali, its sensitivity against acids and mercuric chloride.

Plasmalogen can be determined colorimetrically on the basis of the test with Schiff's reagent (54, 55, 56).

Properties of acetal phosphatides (plasmalogens). Plasmalogens have no definite melting point but become soft at 50° and decompose above 150°. They are insoluble in water, but soluble in dilute aqueous potassium hydroxide. On neutralization of the alkaline solution they precipitate. They are sparingly soluble in cold alcohol, but considerably more soluble in hot alcohol. Methyl alcohol is a better solvent for plasmalogens than ethyl alcohol. They are easily soluble in chloroform but insoluble in ether, petroleum ether, and acetone. When an alcoholic solution of plasmalogen is quickly cooled, the acetal phosphatides precipitate as a waxy, amorphous mass, but on slow cooling they come out in the form of globular aggregates which appear under the microscope as composed of long needles.

Phosphatidic acids. The term phosphatidic acid is used for substances which resemble the monophosphatides in their structure and composition, but which differ from these lipins by the absence of nitrogenous constituents.

Phosphatidic acids were discovered in cabbage leaves by Chibnall and Channon (57). The free acids are soluble in organic solvents such as ether and acetone, but very slightly soluble in water. The sodium salts are soluble in water, but insoluble in ether and very slightly soluble in cold alcohol. The barium, calcium, and lead salts are insoluble in water, but easily soluble in ether. So far, phosphatidic acids have been mainly found in plants.

Plasmalogenic acid, the phosphatidic acid of plasmalogen, has been obtained

by Feulgen and Bersin (51) after saponification of plasmalogen with hot sodium hydroxide.

Pangborn (58) reported the isolation of a phosphatidic acid, cardiolipin, from beef heart. Its presence seems to be essential for the complement-binding activity of beef heart extracts with sera of syphilitic patients. Cardiolipin alone has no complement-fixing power, but mixtures of cardiolipin, cholesterol, and lecithin resemble beef heart extracts prepared for diagnostic use in regard to their serological behavior.

The lipid contains phosphorus amounting to 4.1 per cent, fatty acids, and has an approximate molecular weight of 700. Free cardiolipin is very unstable, but its sodium salt is rather stable.

The separation of cardiolipin from lecithin was accomplished by the transformation into a barium salt which is insoluble in alcohol.

Phosphatides of acid-fast bacteria. Our knowledge of this interesting group of lipins is mainly based on the investigations of Anderson and his associates (1). The chemical composition of these phosphatides differs very markedly from that of the phosphatides isolated from animal tissues. The fatty acids of bacterial phosphatides have already been discussed. Choline and colamine are absent among the hydrolysis products. The N:P ratio in the phosphatides of these micro-organisms is very much lower than 1. Since these phosphatide fractions have not yet been separated into individual components it is still an open question whether they are to be considered as mixtures of phosphatidic acids with a small amount of monoaminophosphatides or as polyphosphatides containing several atoms of phosphorus per atom of nitrogen. The phosphatides from acid-fast bacteria resemble monoaminophosphatides in their solubility. They are easily soluble in ether, and they are precipitated from their ether solutions by means of acetone. Besides glycerol, these substances contain inositol or carbohydrates in ester linkage with the phosphoric acid group. On mild alkaline saponification of the phosphatide fraction of human tubercle bacilli, Anderson, Lothrop, and Creighton (59) obtained a mixture of non-reducing organic P esters which could be fractionated to a certain degree. After dephosphorylation, mannose, inositol, and glycerol were obtained. The hydrolysis of phosphatides obtained from the residues of the manufacture of tuberculin yielded different intermediary phosphoric esters (60). On total hydrolysis glycerol, mannose, inositol, and a glucosazone-forming sugar were found.

The phosphatides of phytomonas tumefaciens (61) do not contain carbohydrate components. The only organic P ester isolated from their hydrolysis product was glycerophosphate. These lipins differ further from those of acid-fast bacteria inasmuch as they contain choline and colamine. The N:P ratio is approximately 1:1. They differ, however, from animal monophosphatides in their content of fatty acids with branched chains.

Anderson and his associates made the interesting observation that the composition of the lipid fractions of micro-organisms which are grown in synthetic media greatly depends on the composition of the medium. For example, Creighton, Chang, and Anderson found phosphatides to be absent in the lipids

of a strain of human tubercle bacilli cultivated on a modified synthetic Long medium in which glycerol was replaced by glucose.

Sphingomyelins. Sphingomyelins are phosphatides in which the sphingosine or a closely related base is bound by an NH—CO linkage to a fatty acid, most commonly to lignoceric acid (n-tetracosanic acid) and by an ester linkage to choline phosphoric acid. Klenk isolated pure stearyl sphingomyelin from human brain in a case of Niemann-Pick's disease (63). In the same case liver and spleen contained a mixture of lignoceryl, nervonyl, and stearyl sphingomyelin (64). Sphingomyelins are insoluble in ether and differ in this respect from the phosphatides of the unsaturated lecithin and cephalin fractions. This property is used for the separation of sphingomyelins from the other phosphatides. It should be emphasized, however, that the insolubility in ether is not a sufficient basis for the identification of a phosphatide fraction with sphingomyelin. Sphingomyelins are soluble in hot alcohol from which they can be crystallized. They are soluble in mixtures of chloroform and methanol and in petroleum ether containing 10 per cent methanol. Sphingomyelins are almost insoluble in acetone and form colloidal solutions with water. Sphingomyelins which were discovered by Thudichum in brain have recently been found in many other organs (1) and in biological liquids such as blood and milk.

The structure of sphingosine. The chemical structure of sphingosine has been elucidated by the work of Levene, of Klenk, and of Carter and their associates. Since the fundamental work on sphingosine by Levene and West (65, 66) and Lapworth (67), its structure has been further clarified in two points: 1) Klenk (68) was able to isolate myristic acid, $C_{14}H_{28}O_2$, from the products obtained after oxidation of sphingosine by means of chromic acid. He concluded from this fact that sphingosine contained a straight chain of 18 carbon atoms (not 17 as had been assumed by Levene). 2) Carter and his associates (69) found that benzoyl sphingosine does not react with periodate, but forms a cyclic acetal with benzaldehyde in the presence of zinc chloride. Since periodate is a specific oxidant for vicinal glycols and since 1,2 as well as 1,3 glycols form acetals it can be concluded that the two hydroxy groups of sphingosine are present in 1,3 position. On the basis of these observations the structure of sphingosine can be expressed by the formula:

$$CH_3 \cdot (CH_2)_{12} \cdot CH{:}CH \cdot CH(OH) \cdot CH(NH_2)CH_2OH$$

Since sphingomyelin does not react with nitrous acid it must be assumed that the amino group of sphingosine is linked to one of the other components of the sphingomyelin molecule. Levene (70) succeeded in isolating lignoceryl hydrosphingosine as a product of the partial hydrolysis of hydrogenated sphingomyelin. Thannhauser and Fraenkel isolated lignoceryl sphingosine (F.P. 90–90.5°) from pigs' liver after saponification with methylalcoholic sodium hydroxide (71). Fraenkel and Bielschowsky (72) and Tropp and Wiedersheim (73) succeeded in isolating lignoceryl sphingosine from pigs' liver and beef spleen without preliminary saponification. The conditions prevailing throughout the isolation procedure were so mild that an artificial formation of lignoceryl sphingosine

was excluded. Substances resembling lignoceryl sphingosine in their general structure (i.e., amides of fatty acids with sphingosine) have been designated as ceramides by Thannhauser and his associates (72). The ceramide group is a constituent of the sphingomyelins as well as of the cerebrosides. The isolation of ceramides from tissues suggests that these compounds are formed in the intermediary metabolism of sphingomyelins and cerebrosides.

Lignoceryl sphingosine shares with sphingomyelin the inertness against nitrous acid and therefore has to be considered as an acid amide.

Thannhauser and Reichel (74) obtained for the first time lignoceryl sphingosine by the enzymatic hydrolysis of sphingomyelin during the incubation with liver extract. The only ceramide which has so far been isolated as a split-product of sphingomyelin is lignoceryl sphingosine. These authors claimed the existence of a sphingomyelin in which the second hydroxy group would be esterified with palmitic acid. In later investigations, it was found that the palmitic acid was not a part of the sphingomyelin molecule, but a part of saturated lecithin which tenaciously adheres to the sphingomyelin fraction (see below).

Ceramides differ from sphingomyelin in their behavior toward ether in which they are soluble. They can be recrystallized from hot acetone (75).

The tests for the purity of isolated sphingomyelin. At present neither sphingomyelin nor any other phosphatide can be reliably identified by determinations of physical constants such as melting points or optical rotation. The purity of a phosphatide can therefore be judged only on the basis of the results of its chemical analysis. Up to 1937, determination of the N and P contents and the result of Molisch's test for carbohydrates represented the only criteria for the purity of specimens of sphingomyelin. Due to the sensitivity of Molisch's test, its negative outcome can be considered as a reliable proof for the absence of cerebrosides. The N:P ratio, however, is not sufficiently sensitive to permit the detection of contaminating monophosphatides with a desirable degree of accuracy.

The introduction of a method for the microdetermination of glycerol into the chemistry of the phospholipids by Blix (76) was therefore a very important addition to the chemical tools available for the examination of the purity of sphingomyelin. By the application of this method, Klenk (77) as well as Thannhauser (78) and their associates were able to demonstrate that all specimens of sphingomyelin which had been considered as pure on the basis of the earlier criteria were in fact contaminated with considerable amounts of glycerin-containing phosphatides, probably of saturated lecithins which have many physical properties in common with sphingomyelin, especially the insolubility in ether.

The removal of these admixtures proved to be an extremely difficult task which has only recently been accomplished by Thannhauser and Benotti (79) for the isolation of sphingomyelin from lung. The isolation of pure sphingomyelin from brain which represents the usual source for the preparation of this lipid has not been accomplished as yet by the exclusive use of fractionation procedures.

The difficulties in the purification of brain sphingomyelin arise chiefly from the presence of large amounts of cerebrosides in addition to that of saturated monophosphatides in the crude sphingomyelin fractions. Klenk (77) as well as Thannhauser (78) found it necessary to subject the crude sphingomyelin fraction to a treatment with alkali which leaves most of the sphingomyelin intact, but which removes the total amount of monophosphatides by a selective hydrolysis. Klenk and Rennkamp (77) refluxed the sphingomyelin fraction with sodium alcoholate. Thannhauser and Benotti (79) found that this treatment was too drastic inasmuch as it destroyed a considerable part of the sphingomyelin. They achieved the selective destruction of the monophosphatides by shaking the crude sphingomyelin for 24 hours with aqueous N sodium hydroxide at 37°.

Nieman (80) recently succeeded in isolating from the hydrolysis products of brain protagon an ether of sphingosine with a tetradecylic alcohol. Nieman's findings recall the old observations by Thudichum who found among the hydrolysis products of sphingomyelin an alcohol with 18 carbon atoms which he called sphingol. Since none of the later investigators confirmed this result of Thudichum this alcohol received no further attention for a long time. The isolation of Nieman's sphingosine ether makes it evident that our present classification of the sphingosine-containing constituents of brain is not yet complete.

Cerebrosides. According to the customary definition, cerebrosides are considered as galactosides of ceramides. The individual cerebrosides were assumed to differ from each other only in respect to the fatty acid components (see page 278). This definition can no longer be considered adequate. 1) Lesuk and Anderson (22) isolated from the larvae of cysticercus fasciolaris dihydrophrenosine whose nitrogen-containing component was dihydrosphingosine. Carter and Norris (81) found dihydrosphingosine among the hydrolysis products of the cerebrosides prepared from beef brain and beef spinal cord. 2) Several investigators (82, 83, 84) found that the cerebrosides which accumulated in the spleens of patients with Gaucher's disease contained as carbohydrate component glucose instead of galactose. In consequence of these results Klenk and Rennkamp (85) reinvestigated the cerebrosides of normal tissues. They found that the cerebroside fractions of all investigated organs contained small amounts of glucose. Thus there is little doubt that glucosidocerebrosides are normal cell constituents as well as galactosidocerebrosides. 3) There is evidence of the presence in tissues of a new group of phosphorus-free carbohydrate-containing lipins, the gangliosides, which differ in their chemical structure from the cerebrosides. E. Walz (86) described in 1927 the presence in beef brain and beef spleen of a cerebroside fraction which differed from the known cerebrosides by the purple color of the Bial test and by its high sensitivity against acids. After heating for a short time with 16 per cent sulfuric acid the formation of a black flocculent precipitate of humin substances was observed. The observations of Walz on normal brain were later confirmed and extended by Blix (87) who found that the new cerebroside fraction gave a positive test for hexosamines with Ehrlich's dimethylaminobenzaldehyde reagent.

In 1935 Klenk (88) reported that relatively considerable amounts of similar

substances could be found in the crude sphingomyelin fractions isolated from the brain in cases of Niemann-Pick's disease. According to Klenk (89) very large amounts of this fraction accumulate in the brain in Tay-Sachs' disease (infantile form of amaurotic idiocy). The normal cerebrosides are totally replaced by this fraction.

Neuraminic acid. In 1941, Klenk (90) succeeded in isolating a hydrolysis product of the new cerebroside fraction in crystallized form. This hydrolysis product for which Klenk introduced the name neuraminic acid represents the group responsible for the characteristic color reactions of the fraction and for its charring on treatment with dilute mineral acid. According to the results of the elementary analysis the substance has either the formula $C_{10}H_{19}NO_8$ (molecular weight 281.16) or $C_{11}H_{21}NO_9$ (molecular weight 311.24). It titrates as a monobasic acid and contains its nitrogen in form of a primary amino group. It is levorotatory in aqueous solution ($\alpha_D = -54 \cdot 91°$). The substance decomposes at 200° without melting point. It is easily soluble in water, only little in methyl alcohol, and insoluble in ethyl alcohol and ether.

On being heated with dilute mineral acids, the solution soon acquires a brown color. During prolonged heating, a black flocculent precipitate appears. The substance, however, is stable against boiling at neutral or alkaline reaction.

Neuraminic acid does not reduce alkaline copper solution. It gives a positive ninhydrine test. On boiling it with Bial's reagent an intense red color develops. After heating it with Ehrlich's reagent in a paraffin bath of 140°, a strong red color appears. The test is strongly positive with 60γ of the neuraminic acid.

Lipoproteins. (91, 92) It has long been known that the extraction of lipins, especially phospholipids, from animal tissues can be carried out successfully only after the denaturation of the proteins. The preparation of phosphatides from egg yolk offers a classical example for this observation. On exhaustive treatment with ether, only traces of phosphatides go into the organic solvent although the neutral fats can be extracted almost completely in this manner. When the yolks are heated with alcohol, however, the total amount of the phosphatides goes quantitatively into the solution and can be easily separated from the protein coagulum.

It is possible to extract from egg yolk with dilute aqueous salt solutions undenatured protein fractions with surprisingly high lipid contents. This fraction can be precipitated and redissolved without much change in its lipid content or its solubility.

Some of the lipid protein complexes migrate in the electrical field like homogeneous substances (93). In order to separate the lipins from their protein carriers, boiling with alcohol or other denaturing procedures are required while the extraction with ether without a preceding denaturation of the proteins does not lead to the dissociation of the lipid protein complex.

It is difficult to reconcile these observations with the assumption that the lipoproteins represent accidental mixtures of lipids and proteins rather than chemical lipid protein complexes. On the other hand, no investigator has succeeded as yet in isolating a lipid protein complex in which the lipid fraction

was homogeneous. The lecithovitallin from egg yolk which has recently been reinvestigated by Chargaff (94) contained approximately equal amounts of lecithin and cephalin.

Liponucleoprotein complexes recently isolated by ultracentrifugation from extracts of normal chicken embryos and from infectious extracts of Rous chicken tumor I contained likewise mixtures of various lipids (95, 96). The same holds true for a lipoprotein with thromboplastic effects isolated from lung tissue (92, 93, 94).

The heterogeneity of the lipid components renders it very difficult to attempt a chemical interpretation of the experimental observations. In this respect, the chemistry of the lipoproteins faces a much more complicated problem than that of many other conjugated proteins such as nucleo-, chondro-, or chromoproteins which are characterized by homogeneous prosthetic groups. Additional difficulties are caused by the great instability of the lipoproteins which has so far prevented any attempt of a finer fractionation of the crude products. It is obvious that, under these circumstances, the experimental facts do not as yet provide a basis for a discussion concerning the forces responsible for the associations between lipins and proteins in the complexes described in this chapter.

II. Recent aspects of the physiology of the lipins. In the studies of the past few years, the chemical functions of the phospholipids in metabolism and nutrition have received more attention than the biological significance of their physicochemical properties for cell permeability and other functions of the cell membrane (1).

Sinclair's investigations (2, 3, 4, 5), especially his observations on the rapid incorporation of ingested elaidic acid (fed as elaidin) into the phospholipid fraction of organs, suggest that the phospholipids have an important rôle in fatty acid transport and fat metabolism. Artom and his associates (6, 7) came to similar conclusions on the basis of their observations that the injection of iodinized fatty acids was followed by the appearance of iodinized fatty acids in the phospholipid fractions of liver and red globules. In addition, Artom (8) found a very considerable temporary increase in the amount of liver phospholipids in dogs which had been fed with large quantities of neutral fats.

Phosphatides are possible precursors of choline and therefore are at least indirectly connected with its functions such as the transmethylation and lipotropic action. Investigations by Engel (9), Stetten and Grail (10), and Fishman and Artom (11) have demonstrated that the amounts of phospholipids, especially those of choline-containing phospholipids, are considerably diminished in choline-deficient rats; the addition of choline to the diet compensates this effect in very young, but not in older animals. Fishman and Artom assume that choline deficiency is not the only factor responsible for the decrease of liver phosphatides in rats under experimental diets. According to these authors, the response of very young animals to choline can be explained by the assumption that weanling rats have a sufficient stock of the hypothetical additional factors.

Similarly the metabolism of cephalin might be involved in that of its compo-

nents such as colamine and inositol. It was demonstrated by Stetten (12, 13) that colamine in the presence of methyl donors, especially methionine, prevents the development of fatty livers. Fishman and Artom (11) found, however, that the addition of colamine or other non-choline substances with lipotropic activity to a choline-deficient diet had no effect on the amounts of liver lecithin although it increased those of the non-choline phospholipids.

The marked lipotropic effect of inositol has been observed in several laboratories (14).

Beveridge and Lucas (15) pointed out that it is difficult to interpret the influence of the diet on the amount of the total liver phosphatides due to the considerable variations of the chemical composition of liver tissue. They found that neither the removal nor the addition of choline, inositol, corn oil, or of the non-essential fat from the diet affected the total relative amounts of phospholipids in rat liver if the calculation was based on the dried defatted liver tissue. These observations, however, would have no bearing on the influence of dietary constituents on the mutual proportion of choline-containing phosphatides and cephalins.

Much of the evidence obtained in experiments on intact animals supports the view that the digestive tract and the liver represent the most important organs in the metabolism of the phospholipids. The relatively high phospholipid content of the lung has led to the question if this organ has an essential function in the lipid metabolism of the organism. MacLachlan (16) has studied the problem on fasting mice in which a rapid mobilization of fats takes place. No significant change in the phospholipid content of the lungs was observed under these conditions. On the other hand, there is evidence that the formation of phospholipids is not limited to the specific activity of liver and intestinal tissue, but represents a more general cell function. It has been demonstrated (17) that a number of isolated tissues (brain, kidney, liver, intestines) incorporate radioactive phosphorus into their phospholipids.

Influence of hormones on the metabolism of phospholipids. The removal of the hypophysis and thyroid glands in dogs is accompanied by a considerable increase of the amount of all blood lipids (18).

Entenman, Lorenz, and Chaikoff (19, 20) demonstrated that the injection of estrogens into male or female immature chickens causes a large increase of all blood lipids. This effect is undoubtedly the main reason for the increase of the blood phosphatides already observed by earlier authors (21, 22) in the laying hen. Since it could be demonstrated (23) that liver slices from birds which had received estrogens are capable of incorporating radiophosphorus at a higher rate into their phosphatide fraction than slices from untreated birds, it would appear that the liver is the site of the increased phospholipid formation in laying birds.

The phospholipid contents of the pig in various stages of its embryonic development have been studied by Gortner (24). He observed a steady decrease of the amounts per gram of dry weight during the gestation. This behavior differs from that of the rabbit embryos (25) in which a strong increase of the phospholipids was found during the development.

Experiments with P_{32}. Since recent comprehensive reviews of this field have been given by Hevesy in Annual Reviews of Biochemistry (1940) (26) and by Chaikoff in this journal (1942)(27), only investigations which have since been carried out will be discussed in this article.

Investigations by Fishler, Entenman, and Chaikoff (28) furnished strong evidence that the liver represents the only site of formation of the plasma phospholipids. These authors administered radioactive sodium phosphate intraperitoneally to hepatectomized dogs. It was found that the amounts of radioactive plasma phospholipids at several intervals up to 6 hours after the injections was only very small in comparison to those found in the control animals. Despite their inability to form plasma phosphatides, the hepatectomized animals were still capable of synthesizing phosphatides at a normal rate in kidney and intestines.

Interesting new observations concerning the fate of the plasma phospholipids were obtained by Zilversmit, Entenman, Fishman, and Chaikoff (29) and by Reinhardt, Fishler and Chaikoff (30). The first group of investigators found, in agreement with earlier observations by Hahn and Hevesy (31), that radioactive phospholipids, when injected intravenously, are rapidly and to a large part removed from the plasma. The larger part of the injected plasma phospholipids could be recovered from various tissues such as liver, spleen, intestines, kidney, and red globules. The second group of authors found that a portion of the radioactive phospholipids reaches the lymphatic channels and was found in the lymph of the thoracic duct. The high rate of the passage of the phospholipids from the bloodstream through the tissues to the lymph channels and back to the bloodstream leaves no doubt that the phospholipids permeate the capillary membranes as such. Since the phospholipids which passed into the thoracic duct are returned to the blood, the observations discussed above establish the existence of a partial "internal circulation of the phospholipids." Apart from the physiological significance of the passage of the phospholipids through the capillaries it appears that the possibility of such an internal circulation must be generally considered in the interpretation of results obtained by the use of tracersubstances in intact animals.

Patterson, Keevil and McHenry (32) found that in choline-deficient rats the turnover of phospholipids was smaller than in normal animals. These results substantiate the conclusions reached by Stetten and Grail (10) on the basis of phospholipid determinations in livers of choline-deficient rats.

As a general summary of the progress achieved in the physiology of phospholipids by the use of tagged molecules it can be said that tracer substances were used like tracer bullets in order to lighten the pathway and the location of the tagged substances in the organism. However, the studies with P_{32} have not so far contributed to our knowledge of the chemical mechanism of the intermediary metabolism of the phospholipids. This will be accomplished only by the chemical isolation of the intermediary products.

Enzymatic hydrolysis of monophosphatides. Our knowledge of the phospholipid-splitting enzymes is still rather fragmentary. The available data concern mainly the enzymes involved in the hydrolysis of lecithin.

In analogy with other enzyme reactions, the hydrolysis of the four ester linkages of lecithin is achieved by different specific enzymes. It has been found that the enzyme systems—and, in consequence the intermediary pathways—of the breakdown of lecithin are different in different organisms.

1) *The lecithinase of clostridium Welchii* (*33*). This microorganism contains an enzyme capable of hydrolyzing the linkage between the glycerol and the phosphoric acid groups of lecithin. Its action results in the formation of phosphoryl choline and diglycerides from lecithin. The enzyme is activated by calcium ions and inhibited by phosphate, fluoride, and citrate ions. It appears to be identical with the alpha toxin which is responsible for the hemolytic and necrotic effects of type A culture filtrates.

2) *Lecitholipases.* a) Enzymes liberating *one* of the fatty acid groups. Lecitholipases which are capable of hydrolyzing one of the fatty acid ester linkages have been found only in the venoms of various snakes and insects (34, 35, 36, 37). The action of these enzymes leads to the formation of lysolecithins which contain only one fatty acid. It seems that these enzymes hydrolyze in preference the ester bond of unsaturated fatty acids in the lecithin molecule.

Slotta and Fraenkel-Conrat (38) reported the crystallization from rattle snake venom (Crotalus T. Terrificus) of a protein possessing the hemolytic and neurotoxic effects in the same quantitative proportions as the crude venom. It can be assumed that this protein is identical with the lysolecithin-forming enzyme. Fairbairn (39) developed a method for the quantitative determination of the lecithin-hydrolyzing enzyme present in the venom of the cotton mouth moccasin (Agkistrodon Piscivorus L.). He found, in addition, that the enzyme acts only on lecithin and cephalin, while cerebrosides, sphingomyelin, acetal phospholipids and lysophosphatides are completely resistant.

b) Enzymes liberating both fatty acid radicals from lecithin. Schmidt, Hershman, and Thannhauser (40) isolated α glyceryl phosphoryl choline from beef pancreas which had been incubated for 3 hours at 37°. The simultaneous disappearance of a large part of the preformed phosphatides suggested the assumption that the isolated diester originated from the enzymatic hydrolysis of lecithin. In 1935 Contardi and Ercoli (41) obtained by incubation of lysolecithin with rice bran a product which apparently consisted of glyceryl phosphoryl choline in impure form. (See supplement.)

Pure α-glyceryl phosphoryl choline is soluble in water and alcohol, but insoluble in acetone, ether or petroleum ether. The substance isolated by Schmidt, Hershman, and Thannhauser is levorotatory (α_D^{20} — 4.8). One of its most characteristic chemical properties is the great lability of the choline ester linkage. When the substance is heated in N HCl on a water bath for 30 minutes, it is practically completely hydrolyzed to choline and glycerophosphoric acid. In this respect α-glyceryl phosphoryl choline closely resembles lecithin whereas phosphoryl choline is very resistant toward acids (42).

The formation of α-glyceryl phosphoryl choline is effected by the action of specific intracellular lecitholipases. These enzymes appear to be firmly bound in the cells since aqueous tissue extracts contain only small fractions of the

enzymatic activity found in suspensions of minced tissues. Pancreatic lipase or duodenal juice are without effect on lecithin or cephalin.

Glyceryl phosphoryl choline is hydrolyzed by alkaline phosphatase, but the rate of hydrolysis is approximately 150 times slower than that observed with glycerophosphate as substrate.

At present, glyceryl phosphoryl choline represents the only substance which has been identified as an intermediary product of the mammalian metabolism of lecithin. Evidence has been obtained that glyceryl phosphoryl choline is found not only in the pancreas but also in other organs, especially in the mucosa of the digestive system. It can be assumed that glyceryl phosphoryl choline is the intermediary substance of lecithin metabolism, especially of its enzymatic disintegration in the intestinal mucosa. On the other hand, the assumption that glyceryl phosphoryl choline might be resynthesized to lecithin in the organism would furnish a simple explanation for the rapid incorporation of ingested fatty acids into the phosphatide fraction (2, 3, 4, 5).

The determination of lipins in tissues. The extraction of the wet tissue according to Bloor (43) is still the basis of the determination of the total lipins and of the individual lipin fractions. This precedes all other analytical procedures. For the determination of the total phospholipids the alcohol-ether extract is evaporated to dryness under reduced pressure or in an atmosphere of N_2; the dry residue is extracted with a small amount of petroleum ether from which the phospholipids can be precipitated by acetone and magnesium chloride and determined oxydimetrically after treatment with chromic acid. The reviewers prefer to determine the total P directly in the petroleum ether extract because the results obtained with this technique are not influenced by the presence of cerebrosides. Gortner (44) recently came likewise to the conclusion that the results obtained with P determination are more consistent than those obtained with the oxydimetric method.

Folch and Van Slyke (45) described a different extraction procedure for the plasma lipids by which certain disadvantages of Bloor's procedure (such as the contamination of the final petroleum ether extract with non-lipid substances) can be avoided. The lipids are precipitated together with the proteins by colloidal iron in the presence of magnesium sulfate. Finally, they are extracted from the washed precipitate by an alcohol-ether mixture at room temperature.

Various analytical procedures for the quantitative estimation of the individual phosphatide fractions have been devised. When they are combined with the determination of the total phospholipids a fairly complete quantitative partition of a given phospholipid mixture is obtained. It should be pointed out, however, that the results obtained with different schemes of the partition show some discrepancies which cannot be explained completely as yet. One of the difficulties encountered in this field arises from the necessity of hydrolyzing the lipids prior to the chemical analysis. Despite the fact that the general behavior of the lipins toward hydrolyzing agents is fairly well known, the conditions required for the quantitative hydrolysis of individual components of the various fractions have not as yet been studied sufficiently to exclude errors due to incomplete hydrolysis.

1) The amount of amino nitrogen in phospholipid mixtures corresponds to the total amount of cephalins. The Van Slyke method is therefore widely used for the determination of cephalin in phospholipid mixtures. It must be emphasized, however, that unsaturated fatty acids evolve considerable amounts of inert gas with nitrous acid (45). Folch, Schneider and Van Slyke (45) found only very small amounts of cephalin in the serum lipids when they used the precaution of hydrogenating the lipids before the determination of the amino nitrogen. Entenman and Chaikoff (47) obtained similar results by means of choline determinations in the plasma lipids. It appears that amino nitrogen determinations in lipid mixtures should be carried out after the removal of the fatty acids by a suitable method of hydrolysis.

2) A micro method for the determination of the two known component nitrogen groups of cephalin, namely, ethanolamine and serine, has recently been reported by Artom (48). It is based on the quantitative liberation of the nitrogen of either substance in the form of ammonia during the oxidation with periodate. When this method is combined with the quantitative separation of both substances by the selective adsorption of colamine on permutite the amounts of either substance can be determined. In many tissues the sum of ethanolamine and serine as determined according to Artom's method agrees with the values for the total cephalin obtained by other methods. In some organs such as brain, kidney, and lung the figures obtained with the periodate method are too high (probably due to the presence of sphingolipids in considerable amounts). It should be mentioned that Artom found considerable amounts of cephalins in blood plasma, contrary to the results obtained with some techniques reported above. (See supplement.)

3) The sum of the choline-containing phospholipids (lecithins and sphingomyelins) can be estimated by the determination of the choline obtained after refluxing the lipid mixture for 2 hours with saturated barium hydroxide (49). The best method for the determination of choline appears to be the spectrophotometry of its reineckate in acetone solution at 327mμ (49). Amounts between 50 and 400γ can be determined with an error of 5 per cent.

Taurog and his co-workers (50) achieved the separation of the choline-containing from the non-choline-containing phosphatides of liver by adsorption of the total phosphatides on magnesium oxide and selective elution of the choline-containing phospholipids by methanol.

4) The sum of the glycerol-containing phosphatides (lecithin and glycerol cephalins) is obtained by glycerol determinations in the dried phospholipid fractions according to Blix (51). This method is analogous to the micro methoxyl determination of Vieböck and Brecher (52).

Attempts have been made to determine the glycerol in lipid extracts by periodate oxidation (53, 54). The application of this principle to the analysis of fats appears to give reliable results. In the case of phospholipids, the use of periodate for the analysis of glycerol incurred serious difficulties: 1) the presence in the hydrolysate of interfering substances such as colamine and serine, and 2) the fact that the usual procedures for the hydrolysis of phospholipids do not lead to the

liberation of glycerol as such, but to the formation of a mixture of α and β glycerophosphoric acid; the latter does not react with periodate and thus escapes determination. It should be emphasized that α and β glycerophosphate, rather than glycerol, should be used as test substance whenever procedures for the determination of glycerol in phospholipid mixtures are to be checked.

5) A convenient micro method for the quantitative partition of phospholipid mixtures into monophosphatides and sphingomyelin has been developed by Schmidt, Benotti, and Thannhauser (55). When lecithin or the cephalins are incubated with N potassium hydroxide at 37° for 15 hours the total amount of their phosphorus groups becomes soluble in dilute acids, while the phosphorus of sphingomyelin remains insoluble under these conditions.

6) A direct microdetermination of sphingomyelin as acetone-insoluble reineckate has been described by Thannhauser, Benotti, and Reinstein (56). The sphingomyelin reineckate includes that of hydrolecithins which have recently been found to be present in several tissues such as brain and lung.

7) A method for the micro determination of cerebrosides has been developed by Brückner (57). It is based on the colorimetric determination of galactose by means of orcinol and sulfuric acid in the hydrolysate of the lipid extracts. In regard to its specificity it is superior to the earlier reductometric methods (58); it should be kept in mind, however, that the figures obtained by Brückner's method do not include the glucose-containing cerebrosides recently found in Gaucher organs and also in normal tissues. (See supplement.)

Remarks. It can easily be seen that by a suitable combination of several of the procedures just discussed, it will be possible to achieve a rather complete quantitative partition of the phospholipids. It must be emphasized, however, that the accuracy of figures obtained by difference should be carefully examined in each case. In the majority of tissues, only the total lecithin and the total cephalin fractions are so large that errors of the individual determinations do not seriously interfere with the calculation of the differences.

Representative figures of the concentration of phospholipids in various tissues (48, 59, 60) and in isolated nuclei (61) have been reported by several authors.

Peters and Man (62, 63) have recently reported their experiences concerning the amounts of blood phospholipids in humans under normal and pathological conditions. They found that the figures for the phospholipid P in normal serum range between 6.1 and 14.5 mgm. per 100 cc. High values are often observed in thyroid deficiency and low values in hyperthyroidism although normal concentrations may be found in either condition. In patients with kidney diseases the concentration of phospholipids in the serum is usually elevated. In both groups of diseases, the ratio between the amount of cholesterol and phospholipids is not altered in comparison to that found in normal serum.

III. Lipidoses. The group of metabolic disorders designated as "lipidoses" is a heterogenic collection of disorders. To combine this group under the name "lipidoses" has been generally accepted since fat-like substances of different chemical constitution and properties are found which accumulate in reticulum cells and histiocytes. The mechanism leading to such an accumulation of lipids

within these cells has not been definitely ascertained. In explaining this mechanism theories have been advanced which are based on the faculty of reticulum cells and histiocytes to take up lipids from the blood stream as well as to synthesize and retain these substances within the cell.

A. Xanthomatoses. For the determination of cholesterol and its esters in body tissues and serum, the digitonin precipitation of free cholesterol (Windaus, 1) is used. On the basis of such a precipitation a method has been described by Schönheimer and Sperry whereby only small quantities of material are necessary (2). This method is generally used for the estimation for cholesterol and its esters in tissues and serum. The normal serum values in adults range between 190 and 240 mgm. per cent for total cholesterol, between 30 and 60 mgm. per cent for free cholesterol, and between 100 and 180 mgm. per cent for cholesterolesters. The cholesterolesters should constitute between 60 and 70 per cent of total cholesterols in serum.

The foam cell, the cholesterol imbued reticulum cell, characteristic of the various types of xanthomatosis may originate

(I) From an increased uptake of cholesterol from a serum in which cholesterol is pathologically accumulated, or

(II) From an increased synthesis of cholesterol within the cell without increased supply from the blood stream.

I. In basing the origin of the foam cell on the increased uptake of cholesterol in the reticulum cell, it is presumed that the cholesterol content of the supplying tissue fluids (blood serum or local tissue fluids) is increased.

An accumulation of serum cholesterol (Hypercholesteremia) may result from:
a.) A decreased destruction of cholesterol. Thus far no enzyme capable of splitting the terpenlike ring-system has been isolated from mammalian tissues. The chemical changes, which occur in the intermediary metabolism of the cholesterol molecule take place in the side chain of that molecule, such as esterification of the alcoholic hydroxyl group, hydrogenation and oxydation on the sterol ring as well as of the side chain. It is not definitely known whether the sterol-sex hormones are metabolites of cholesterol or the result of sterol synthesis. Cholesterol derivatives in minimal quantities have been isolated from animal organs. It was suggested that cholestenone was present in deposits of cholesterol in the arterial wall (3, 4). Hydrocholesterol was isolated from the liver and from the serum of pregnant mares (5). Dicholesterylether is present in the spinal cord of the ox (6). Cholestenone resulted from the action of proactinomyces upon cholesterol (7).

A destruction of the cholesterol skeleton was concluded from negative cholesterol balance experiments (8, 9, 10, 11, 12, 13). In these balance experiments cholesterol was determined as cholesterol-digitonid. From the fact that the expected amount of cholesterol did not precipitate with digitonin it cannot be concluded that the cholesterol skeleton was destroyed in the intermediary metabolism. The sterol nucleus may well be intact while slight oxidative or reductive changes may have resulted in a sterol unprecipitable with digitonin or not giving the Liebermann-Burchard test. Since it has been shown that

bacteria present in the intestinal tract transform cholesterol to a sterol not precipitable by digitonin (14, 15), it may be concluded that the deficit of cholesterol in balance experiments in animals and in men is due rather to bacterial action in the intestines upon cholesterol than to a disintegration of the sterol ring in the intermediary metabolism. On the basis of our present knowledge an accumulation of cholesterol in the blood serum or in tissues cannot be explained by a decreased destruction of the sterol ring in the intermediary metabolism.

b.) The *accumulation of cholesterols* in the organism may be due to an *impaired excretion* of this substance. Such a disturbance with resulting retention of cholesterol may be functional or mechanical. As far as we know cholesterol is excreted by the liver via the bile capillaries (16, 17) and probably also by the cells of intestinal mucosa (18, 19, 20). A disturbance of such an excretory function may result in cholesterol retention in the serum. It is not likely that at the onset of a functional disturbance anatomical changes of the affected organ are detectable. A diminution of cholesterol excretion may be limited to cholesterol alone; it may also be accompanied by a diminution of other bile constituents like bilirubin and bile acids, i.e., it may occur with or without jaundice. (Hypercholesteremia in hepatitis, essential xanthomatosis of the hypercholesteremic type, see p. 299).

c.) It is evident that *mechanical obstruction* of the bile passages, due to stone, inflammatory or tumerous obstruction, results in a retention of all bile constituents and consequently cholesterols. The serum of patients suffering from mechanical obstruction of the bile ducts does not usually show extremely high cholesterol figures (usually twice that of normal). The ratio of cholesterol: cholesterolesters (increase of free cholesterol, decrease of cholesterolesters) is only altered if acute or chronic damage of the liver cells accompanies the mechanical obstruction (21, 22, 23, 24). (Hypercholesteremia in obstructive jaundice.)

d.) The cholesterol content of the blood serum may be increased without functional or mechanical impairment of cholesterol excretion in cases where *hyperlipemia* (*creamy serum*) occurs. Hyperlipemia may be the result of a disturbed metabolism of neutral fat or may be due to an impaired transportation or deposition of neutral fat. Whenever neutral fat increases in the serum, cholesterol accompanies the neutral fat and results in an increase of free and ester cholesterol in the serum. In such cases the appearance of foam cells is due to an increased uptake of cholesterol from the serum and is observed in different organs especially in skin, spleen, liver and lungs: Secondary hyperlipemia with xanthomatosis due to diabetic hyperlipemia (25, 26, 27). Secondary hyperlipemia with xanthomatosis in chronic pancreatitis (28, 29). Secondary hyperlipemia with xanthomatosis in glycogen storage disease (Von Gierke's disease) (30, 31, 32). Idiopathic familial hyperlipemia with secondary xanthomatosis (33, 34, 35). Hepatosplenomegaly with hyperlipemia and xanthomatosis type Bürger Grütz (36).

e.) In cases of *hypothyroidism* the cholesterol content of the serum is high. The reason for the increase of serum cholesterol is not known. Thyroid medication reduces the cholesterol level of the serum. In rare cases of hypothyroidism

with very high serum cholesterol levels, foam cells with xanthoma formation may originate in the skin (37).

f.) A local accumulation of cholesterol with foam cell formation may occur in any type of *organs where cells undergo destruction* by inflammatory processes or tumor growth. In such circumscribed areas the cholesterol derived from necrotic tissue may appear in the detritus as cholesterol crystals or may be taken up by reticulum cells, thereby changing their appearance to foam cells. (Xanthoma cells in inflammatory tissue; osteomyelitis; osteitis fibrosa cystica disseminata; xanthomatous transformation of the mesentery; xanthoma cells in tumors.) (38)

II. *The second mechanism in the origin of xanthoma cells* is probably initiated by intracellular disturbance of enzymes concerned with the cholesterol metabolism. As already mentioned enzymes capable of splitting the sterol skeleton are not known to be active in the intermediary and cellular metabolism of animals. For this reason an accumulation of cholesterol due to an enzymatic disturbance is not likely to be the result of a decreased cholesterol catabolism, but rather to an increased anabolism, i.e., synthesis of cholesterol. It has already been shown in the earlier investigations of cholesterol metabolism that the sterol-ring system is constantly synthesized in the mammalian organism (39, 40, 41, 42). Schönheimer, feeding materials containing deuterium, demonstrated that the small molecules, of 2 and 3 carbon atoms, which may be derived from all three food constituents (proteins, carbohydrates and fats) are the basis of cellular sterol synthesis (43). From the experiments of Bloch, using labeled acetic acid, it is evident that considerable quantities of cholesterol are synthesized from a molecule as small as acetic acid (44). The question arises which organs and which cells are capable of cholesterol synthesis. This question cannot be definitely answered. It is probable that every growing cell during maturation is capable of synthesizing cholesterol but this function seems to be maintained to a higher degree in reticulum cells in which the functional possibilities of embryonal cells especially of embryonal fat cells to form all kinds of lipids are preserved (45, 46). In the fully developed organism the liver seems to have a special part not only in the excretion but also in the performance of the synthesis of the sterols. The experiments of Thannhauser, Enderlen and Jenke on dogs with bile fistulas demonstrated that the synthesis of the sterol-skeleton of bile acids occurs as a biological synthesis in the liver (16). In further experiments (47) it was shown that after liver extirpation in dogs the serum cholesterol is not decreased after 24 hours. The lowest values of cholesterol, however are found in the serum of patients suffering from acute yellow liver atrophy (48). This observation supports the theory that the liver plays an important part in the formation of cholesterol in the mature organism. It is suggestive to draw an analogy of the behavior of cholesterol and of porphyrins in the metabolism. Both substances consist of ring systems, a terpen ring on the one hand and pyrrolrings on the other hand. Both ring systems are formed in the cells, but neither the terpenring nor the pyrrolring is split by enzymatic disintegration in animals. The mammalian organism is apparently fit to synthesize ring systems

but is not capable of splitting these ring systems after they are formed. The enzymatic synthesis of sterols and pyrrols is apparently performed by the same organ cells, namely, the reticulum cells and histiocytes. Since the greatest accumulation of reticulum cells is found in the liver, it seems understandable that this organ is the outstanding organ involved in the formation of cholesterol. The reticulum cells dispersed in other organs are supposed to be capable of performing the same functions but the actual magnitude of their part in cholesterol synthesis under normal conditions is not well known. We only are aware that the cholesterol content of the serum in acute yellow liver atrophy is markedly decreased but nevertheless cholesterol in the serum is always present indicating that cholesterol is also formed outside of the liver. For these reasons different clinical syndromes may occur under pathological conditions whether the increase of cholesterol formation occurs in the liver or is performed outside of the liver, manifesting itself, in the latter case, as a systemic disorder of the reticulum cells and histiocytes.

a.) *Increased cholesterol formation in the liver.* In the liver, the cholesterol excretion in the bile and the release of newly formed cholesterol into the blood stream seems to be functionally related. An increase of cholesterol formation in the liver may therefore involve these functions in such a way that in case more cholesterol is formed and released into the blood stream more cholesterol is excreted into the bile fluid. However, the physiological range of the excretory functions of cholesterol in the liver is apparently limited since the concentration of cholesterol in the bile is low and varies but little (16, 49). For this reason increased cholesterol formation in the liver must lead to a considerable accumulation of cholesterol in the blood serum, without primarily involving the other functions of this organ. It is understandable that as long as the liver parenchyma is undamaged the increased amount of cholesterol produced and released into the blood stream will appear as cholesterolester (21).

The clinical syndrome designated by Thannhauser and Magendantz as "primary essential xanthomatosis of the hypercholesteremic type" may be explained on the basis of such a pathogenesis initiated by an increased cholesterol formation in the liver. It is manifested by an accumulation of cholesterol in the serum since the physiological facilities for cholesterol excretion in the liver apparently cannot adjust themselves to the increased supply.

This syndrome is characterized by 1) xanthoma formation of the skin (xanthoma tuberosa et plana); 2) xanthoma of the tendons whereby nodes are produced consisting of cholesterol-containing foam cells and fibrous tissue; 3) xanthoma of the intima of blood vessels and of the endocardial linings of the heart. The incidence of this syndrome in families as recessive hereditary stigma is of great interest. In members of the same family the complete syndrome may be present while in others only hypercholesteremia may be found. (Incomplete form, form fruste) (50).

In contrast to other types of hypercholesteremia the serum in this group of cases does not contain increased amount of neutral fat and is therefore not creamy in appearance. This significant difference is due to the different mecha-

nism resulting in hypercholesteremia. It is suggested that in "essential xanthomatosis of the hypercholesteremic type" the hypercholesteremia is caused by an increased formation of cholesterol in the liver not compensated by an increased excretion, while hypercholesteremia associated with an increase of neutral fat (creamy serum) is a phenomenon due to a disturbance of the deposition or transportation of neutral fat as cholesterol accompanies neutral fat wherever it travels and accumulates in the organism. In the liver of cases of "essential xanthomatosis of the hypercholesteremic type" anatomical changes may not be detectable in the beginning of the disorder. This is understandable, since a functional disturbance of the balance between cholesterol production and cholesterol excretion is assumed to be the pathogenetic factor for this type of essential xanthomatosis. If, however, in later stages of the disorder anatomical changes of the liver occur, such changes do not consist of foam cell formation within the liver tissue itself but they appear clinically and anatomically as features of a slowly developing biliary cirrhosis. It has been suggested that this special type of biliary cirrhosis be designated "xanthomatous biliary cirrhosis" (50) because its outstanding features are hypercholesteremia with xanthoma formation of the skin of the tendons and of the inner linings of blood vessels. In some of these cases also the inner linings of the bile ducts and of the gall bladder are covered with xanthomatous lesions. The chemical analysis of the serum in these cases shows an increase of the total cholesterol and also an increase of lecithin to 3–6 times of the normal values. The cholesterolesters are prevalent. The neutral fat however is normal or only slightly increased. The bilirubin is considerably elevated in proportion to the jaundice which has been present for many years. In the later stages of the disease, concurrent with the slow progress of the liver damage and the slowly developing cirrhosis, the cholesterol values decrease gradually and the cholesterol:cholesterolester ratio changes in favor of the free cholesterol. Dehydrocholesterol which is present only in small amounts in normal serum is found to be increased (51).

b.) *Increased cholesterol formation in reticulum cells and histiocytes.* Pinkus and Pick (51) were the first to discover that the fat substances in the foam cells are cholesterol and cholesterolesters. They advanced the theory that "cholesterol infiltration of certain cells takes place because of an increased cholesterol supply from the blood." There is however a clinically and anatomically well defined group exhibiting xanthoma formation without increased cholesterol supply from the blood presenting normal cholesterol values of the serum. Such an occurrence cannot be explained by the assumption of an infiltration of cholesterol due to an increased supply. Waldeyer (45) has already suggested that the xanthoma cell is an embryonal cell capable of forming different kinds of lipids, which are retained within the cell and only released by disintegration of these cells. Since reticulum cells and histiocytes retain the functional possibilities of embryonal cells it can be assumed that they also are capable of forming various kinds of lipids and also of cholesterol. It is conceivable that in these cells a disturbance of the intracellular enzymatic systems concerned with the formation of cholesterol may result in an accumulation of cholesterol within the

cell thereby transforming these cells into xanthoma cells (foam cells). It may be understood that also under these abnormal conditions the cholesterol formed in excess is retained within the cell and not released into the blood stream. Such an explanation was applied by Thannhauser and Magendantz (50) to the pathology of a systemic xanthomatous disorder which they designated as "Primary essential xanthomatosis of the normocholesteremic type" singling out the normal cholesterol content of the blood as the leading symptom. As the foam cells in this type occur concurrently with a systemic new-growth of granulomatous tissue the disorder was designated by W. Chester (53, 54) as "Lipoidgranulomatosis." Recently the pathologists L. Lichtenstein and H. Jaffe (57) and especially S. Farber (55, 56) designated this group as "Eosinophilic granuloma" because eosinophilic cells as well as giant cells are found in the granulomatous tissue beside the nests of foam cells. In some juvenile cases nests of foam cells may even be absent or very scarce. Whatever designation these authors use they refer to the same disease. It is characterized by a special type of a systemic granuloma in which foam cells singly or in large nests have developed despite the fact that the cholesterol level of the circulating blood is normal. In earlier times before all the features of this syndrome were sufficiently studied considerable confusion was caused by the fact that only one or the other organ may be affected by the disease. Various names referring to the organ involved are found in the literature such as: xanthoma disseminata of the skin, lipid granulomatosis of the bones, Hand-Schuller-Christian syndrome.* They are all singly or in various combinations in the syndrome (essential xanthomatosis of the normocholesteremic type, eosinophilic granuloma, lipid granuloma) are the skin (disseminata type of xanthoma), the skeleton, dura, brain, lungs, pleura, lymph nodes, liver and spleen (50).

While a description of the clinical picture of this systemic disorder and an elaboration of the differential diagnosis in question has no place in this review, three differential diagnostic points, however, should be stressed in distinguishing the hypercholesteremic from the normocholesteremic type of essential xanthomosis.

1.) The skin xanthoma in the hypercholesteremic type are "xanthoma tuberosa et plana" of carotinlike color. The skin xanthoma in the normocholesteremic type are "xanthoma disseminata" of sepia or chamois hue. Both kinds are also different in their location.

2) The organs involved in the "hypercholesteremic type" are the skin, the tendons, the inner layer of blood vessels and possibly the inner layer of the bile ducts. This type occurs often in families. The organs affected in the "normocholesteremic type" (eosinophilic granuloma) are the skin (disseminata type of xanthoma), the skeleton, dura, brain, lungs, lymph nodes, spleen and liver. Tendons and inner layer of the blood vessels are not involved in the "normocholesteremic type." This type of essential xanthomatosis is *not* inherited.

3) If the liver is involved in the "hypercholesteremic" type it differs clinically and anatomically from the liver involvement in the "normocholesteremic"

* The sentence beginning on line 22 (page 301) should read as follows: They are all a part of the syndrome under discussion. The organs which may be involved singly or in various combinations in the syndrome (essential xanthomatosis of the normocholesteremic type, eosinophilic granuloma, lipid granuloma) are the skin (disseminata type of xanthoma), the skeleton, dura, brain, lungs, pleura, lymph nodes, and spleen (50).

type (eosinophilic granuloma). In the hypercholesteremic type, jaundice of the obstructive kind is present lasting many years, biliary cirrhosis and in the late stages even symptoms of portal obstruction may develop. Foam cells are not formed in the liver tissue. In the normocholesteremic type (eosinophilic granuloma) however, in contrast to these findings, jaundice is never present. While the liver disease does not develop to biliary cirrhosis, little grayish nodules of granulomatous tissue are found irregularly dispersed in the liver consisting of granulomatous tissue containing nests of foam cells and also eosinophiles.

A classification of the various kinds of diseases exhibiting xanthoma formation is suggested by Thannhauser (58).

I. Primary Essential Xanthomatosis (Reticular and Histiocytic Cholesterosis)
   A. Primary essential xanthomatosis of the hypercholesteremic type.
      1. Xanthelasma of the eyelids and xanthoma tuberosum et planum
      2. Tendon xanthoma
      3. Xanthoma tuberosum et planum and tendon xanthoma
      4. Xanthoma of the blood vessels and endocardium
      5. Forme fruste of essential xanthomatosis
      6. Xanthomatous biliary cirrhosis
   B. Primary essential xanthomatosis of the normocholesteremic type (eosinophilic granuloma)
      1. Xanthoma disseminatum of the skin
      2. Xanthoma disseminatum and diabetes insipidus
      3. Osseous xanthoma
      4. Schüller-Christian syndrome: Osseous xanthoma (defects in the membranous bones of the skull), exophthalmus and diabetes insipidus
      5. Generalized xanthoma of the normocholesteremic type. (Eosinophilic granuloma) Xanthoma disseminatum; xanthoma of the dura and brain; xanthomatous involvement of the lungs and pleura with consequent pulmonary fibrosis; xanthomatosis of the lymph nodes; hepatosplenomegaly due to scattered nests of xanthoma cells in the spleen and liver

II. Secondary Xanthomatosis Due to Hyperlipemia
   A. Idiopathic (familial) hyperlipemia with hepatosplenomegaly and secondary xanthoma
   B. Secondary xanthomatosis due to diabetic hyperlipemia
   C. Hyperlipemia with secondary xanthomatosis in chronic pancreatitis
   D. Hyperlipemia in glycogen storage disease
   E. Hyperlipemia in lipoid nephrosis

III. Localized Xanthoma Formation in Inflammatory Tissue and in True Tumors
   A. Xanthoma cells in inflammatory tissue
      1. Inflammatory xanthoma of the breast,
      2. Osteomyelitis, osteitis fibrosa cystica disseminata
      3. Xanthomatous transformation of the mesentery (intestinal lipodystrophy of Whipple)

4. Xantholipomas
B. Xanthoma cells in tumors
   1. Nevo-xantho-endothelioma
   2. Xanthomatous polycystic lymphangiomas
   3. Single xanthomatous giant cell tumors
   4. Epithelial tumors with xanthoma cells
C. Xanthoma cells in other conditions
   1. Lipoid proteinosis
   2. Necrobiosis lipoidica diabeticorum

Schönheimer and his co-workers (59, 60, 61, 62, 63) have shown that neither herbivorous nor carnivorous animals absorb plant sterols from the intestines. Animal cholesterol derived from food or from secretion in the bowels is the only sterol absorbed from the intestines. For this reason a diet low in animal cholesterol containing mainly fat of vegetable origin should be given to patients exhibiting xanthoma with hypercholesteremia (64). In cases of "essential xanthomatosis of the normocholesteremic type" a diet low in animal sterols has no influence on xanthoma formation.

B. Niemann-Pick's Disease (Reticular and Histiocytic Sphingomyelinosis). This disorder was first described by A. Niemann in a case reported in 1914. (1) It was L. Pick, in 1927, who differentiated this condition (2) from Gaucher's disease. E. Klenk (3, 4) discovered that the lipid present in the large pale cells was mainly sphingomyelin. These sphingomyelin-loaded cells are found in all organs of the body and are not restricted the lympho-hemopoietic apparatus. Comprehensive studies of the disease are included in the monograph of T. Baumann, E. Klenk, and S. Scheidegger, (5) and S. J. Thannhauser (6). In the presence of Niemann-Pick's disease sphingomyelin determinations in different organs showed a tremendous increase over the normal. However, in the brain the sphingomyelin content was found in the range of normal values (3, 7, 8, 9). The fatty acids yielded after hydrolysis of sphingomyelin of Niemann-Pick organs did not differ from the fatty acids of sphingomyelin prepared from normal organs (10, 11). Sphingomyelin of brain of Niemann-Pick disease however was found to contain only stearic acid in contrast to sphingomyelin of normal brain, which yielded lignoceric, palmitic, and stearic acid (12). Other authors (13, 14) have reported that mainly stearic acid is present but also lignoceric and palmitic acid are found in the sphingomyelin isolated from the Niemann-Pick brain. These studies elaborating the differences of the fatty acids of sphingomyelin cannot be considered as final since it was not known at those times that the sphingomyelin isolated by the methods of preparation generally used is mixed with a glycerol containing monophosphatide (15, 16, 17). This substance was shown to be a hydrolecithin containing palmitic acid (18). As the physical properties of hydrolecithin are almost identical with sphingomyelin its presence in sphingomyelin preparations has eluded discovery. It is therefore not yet proven that sphingomyelin isolated from Niemann-Pick brain may differ from normal sphingomyelin in respect to its fatty acids.

L. Pick's conception (2, 19) of the pathogenesis of Niemann-Pick's disease was based on the idea that sphingomyelin is taken up from the circulating blood and

deposited and stored in the reticulum cells and histiocytes. By this conception it is assumed that the substance deposited and accumulated in a cell is first increased in the serum. The sphingomyelin content of the serum of patients suffering of Niemann-Pick's disease however is normal (5, 20). The hypothesis that sphingomyelin is deposited in the cell as a result of its accumulation in the serum is not substantiated by these analytical findings. Sperry (21), however, is not of the opinion "that a normal lipid concentration in the serum of patients with lipoidosis rules out the possibility that the lipids deposited in tissues were transported there by the blood, it may indicate that the primary lesion is in the cells, where the deposition takes place and is of such a nature as to cause the uptake of lipids from the serum by a sort of vacuum process." This rather unusual conception of a "vacuum process" is unparalleled in cellular metabolism of animals. A deposition and infiltration of reticulum cells and histiocytes with sphingomyelin (22) and with aceton-insoluble lipids obtained from beef brain (23) was only possible by injection of these substances whereby their concentration in the body fluids was abnormally increased.

In analogy with the conception of the pathogenesis of essential xanthomosis of the normocholesteremic type (lipid granulomatosis, eosinophilic granuloma) Thannhauser expresses the opinion that also in Niemann-Pick disease an intracellular enzymatic disturbance of sphingomyelin disintegration or new formation leads to an accumulation of sphingomyelin within the cell.

Knowledge concerning the enzymes involved in the ana- and katabolism of sphingomyelin is very meager. The occurrence of ceramides (fatty acidamids of sphingosin) in organs where also sphingomyelin is found (24) suggests the idea that ceramides are the basic substances from which the synthesis of sphingomyelin proceeds by esterification with phosphorylcholine. Vice versa, ceramides may originate from sphingomyelin by splitting of phosphorylcholine from its molecule. There is however up to the present no experimental evidence of the enzymes involved in such a reaction so that the pathways used for sphingomyelin formation and disintegration are unknown. The methods employed for the estimation of sphingomyelin are described on page 295.

The Niemann-Pick cell is pale and foamy in appearance and forty-five or more microns in diameter (25, 26). There is no new growth of granulomatous tissue as is found in essential xanthomatosis of the "normocholesteremic" type. (Lipid granulomatosis, eosinophilic granuloma.) Concerning the histiogenesis of the Niemann-Pick cell, opinions vary, since not only the reticulo-endothelial apparatus but more or less all the tissues of the body are involved. Bloom (27) expressed the following opinion: "In studying the tissues for the source of lipoid-laden phagocytes, certain genetic relationships between some of the various connective tissue cells of the human organism become apparent. All stages in the mobilization of the embryonic mesenchymal cells and the mature histiocytes well into large lipoid containing histiocytes can be clearly seen. The foam cells develop from the resting endothelial and from the Kupfer cells in the liver, from the reticulum cells of the lymph nodes, the reticulum cells (exclusive of the littoral cells) in the spleen and bone marrow, from the reticulum cells of the medulla of

the connective tissue and the perivascular embryonic cells and phagocytes all over the body."

Chemical analysis of the blood serum in Niemann-Pick's disease shows a normal composition of its constituents (5). The neutral fat is slightly increased but as already mentioned, the concentration of sphingomyelin is within normal limits (28). In the blood smear giant reticulum cells probably containing sphingomyelin were found (5). The ganglion cells of the brain are enormously blown up and show disintegration of the tigroid bodies. All areas of the brain demonstrate the same degenerative process of the ganglion cells (5). Niemann-Pick cells usually do not stain with the customary fat stains, Sudan III or Nile blue sulphate. The Lorrain-Smith-Dietrich technique is used to demonstrate sphingomyelin in tissue cells.

Niemann-Pick's disease has been observed in single cases and also in families. The disease occurs in infants. The infants usually are born at full term and with normal weight. The onset of the disease is insidious; first there is loss of appetite and corresponding loss of weight until the infant becomes thin and emaciated. The abdomen becomes enlarged. Diarrhea and vomiting result in dehydration. Gradually the motor and psychic functions are lost. In the advanced phase of the disease the child is apathetic. The face has a mongoloid expression; the mouth is open and the tongue protrudes. The emaciated extremities have an increased tone and can scarcely move. The infant becomes completely debilitated mentally and physically until death terminates its life between the first and second year. The skin shows a diffuse brownish-yellowish pigmentation. There is also pigmentation of the mucous membranes of the mouth and blue-black spots on the tongue and on the gums (so-called mongolian spots) are present. The skeletal and nervous system as well as all internal organs are involved in the disease and may produce clinical symptoms (20).

C. Gaucher's Disease (Reticular and Histiocytic Cerebrosidosis). Under the title "De l'epithelioma primitif de la rate" in 1882, E. Gaucher described a disease which is characterized by an enlargement of the spleen (1). It was not until 1905 that Brill, Mandelbaum and Libman (2) recognized the involvement of the liver and the osseous system as well as the spleen in this disease. It was thought that this disease was neoplastic in nature. Schlagenhaufer was the first, in 1906, to refer to this disease (3) as a disorder of the lymph-hemopoietic system. Marchand, 1907, in a study of the histology of the disease (4) pointed out that it was neither neoplastic nor simple hyperplasia but resulted from a deposit of some foreign substance. This substance was identified by Lieb in 1927 as cerebroside and was thought to be identical with kerasin (5, 6). Epstein and Lorenz, 1930, referred to Gaucher's disease as analogous to xanthomatosis and Niemann-Pick's disease calling attention to the fact that in these three disorders three different types of lipids are found predominantly deposited in large cells of various organs (7). L. Pick (1933) classified these three diseases as disorders of the lipid metabolism suggesting that the different lipids involved in the special type are accumulated in the blood stream and deposited and stored in the reticulum cells (8). A special form of Gaucher's disease occurring only in infancy was described, 1927,

by Oberling and Woringer (9). The infantile form is not restricted to reticulum cells of the lymph-hemopoietic system, but as in Niemann-Pick's disease involves all organs transforming the resting mesenchymal cells as well as the mature histiocytes and reticulum cells into large cells laden with cerebrosides.

Since L. Pick in his classical description of the lipid disorders (8) advanced the opinion that in Gaucher's disease the lipid accumulation is the result of a metabolic disorder, different theories have been suggested. The focal point of Pick's theory is the assumption that cerebrosides are deposited in the cell from the blood stream. Such an opinion has as a prerequisite that cerebrosides are present in abnormal amounts in the serum of these patients. No one up to the present time, however, has been able to isolate cerebrosides at all from normal serum or from the serum of Gaucher's disease (10, 11, 12). The only reference to the presence of cerebrosides in serum is found in papers dealing with "quantitative" methods of determination of cerebrosides in plasma (13). These methods are indirect methods based on the reduction of sugar liberated by hydrolysis from cerebrosides. However Thannhauser and co-workers (13) as well as Brückner (12) were not able to demonstrate measurable amounts of cerebrosides in normal serum. Correspondingly in the serum of patients affected with Gaucher's disease not more than traces of a substance giving the Molisch reaction could be detected (10, 11). On the basis of these negative findings it seems justified to assume that cerebrosides are not present in measurable quantities either in the serum of normal individuals or in the serum of patients afflicted with Gaucher's disease. A theory explaining the accumulation of cerebrosides in the reticulum cells of Gaucher's disease by an increased transportation and secondary deposition in the cells does not seem therefore to coincide with the actual findings. Thannhauser (14) expressed the opinion that cerebrosides are produced within the same cells, in which they are found. Consequently a metabolic disorder leading to an overproduction and accumulation of cerebrosides must take place within the cell, where this substance originates. For this reason it was suggested that the accumulation of cerebrosides in Gaucher's disease is due to a disturbance of the intracellular enzymes concerned with the ana- and catabolism of cerebrosides; this explanation is analogous to the conception ventured for the etiology of essential xanthomatosis of the "normocholesteremic" type (lipid granulomatosis, eosinophilic granuloma) and for Niemann-Pick's disease, see p. 308.

In animals injections of cerebrosides as well as injections of cholesterol and sphingomyelin produce an increase of the serum level of these substances above the normal and a secondary deposition in the phagocytic histiocytes (15, 16). Such experiments, however, do not permit the assumption that an analogous sequence of events (accumulation above the normal level in the serum and deposition in the reticulum cells and histiocytes) is responsible for the accumulation of lipids in Gaucher's disease or in essential xanthomatosis of the normocholesteremic type or in Niemann-Pick's disease. The lipid cells in these disorders originate while the concentration of the lipids involved in the serum is not higher than that found in normal individuals. The formation of lipid-laden histiocytes after injection of various lipids, however, is analogous in its mechanism to the

xanthoma cell formation secondary to hyperlipemia accompanied by hypercholesteremia. See page 297.

In normal individuals cerebrosides are found in great quantities in brain and nerve tissue and in very small quantities in spleen (17) and lung (18) . It was believed that the cerebrosides accumulated in the organs of patients with Gaucher's disease are identical with kerasin, a galactoside occurring in brain tissue (5, 6). Aghion (19) and especially Halliday, Deuel, Tragermam, and Ward (20) however proved conclusively that the cerebrosides found in the organs of Gaucher's diseases are mainly glucosides since their sugar component fermented with yeast. This finding later was confirmed by Klenk (21, 22) and other authors (23). The question has been raised whether Gaucher's disease results from the formation of an abnormal, unphysiological glucose-containing cerebroside. Klenk, however, demonstrated that the small quantities of cerebrosides present in normal beef spleen yielded both types of cerebrosides, galactosides and glucosides (22). Also in small amounts of cerebrosides isolated from beef lungs both types of cerebrosides were found (24). For this reason it is not permissible to assume that the glucose containing cerebroside found in Gaucher's disease is an abnormal cerebroside. The interesting fact, however, remains that in Gaucher's disease mainly a glucose-containing cerebroside is formed and retained within the cells of the involved organs (spleen, liver, lymph nodes, and bones). The presence of a glucose-containing cerebroside as the main constituent of the lipid accumulated in Gaucher organs is an important reason against the assumption of an increased transportation and deposition of cerebrosides as etiology for this disease, since in brain which is not involved in the cases of adult Gaucher's disease the cerebrosides present are galactosides as in normal brain.

Very little is known concerning the nature of the enzymes producing cerebroside formation and disintegration. A glucoside-splitting enzyme is present in the spleen (25). There is a lack of knowledge concerning the enzymes responsible for the synthesis of cerebrosides. Also the intermediary products occurring in formation and disintegration of cerebrosides are not definitely known. It was, however, suggested that the ceramides (like lignocerylphingosine) being a constituent of both the cerebrosides as well as the sphingomyelin molecule are the basic compounds parting from which the synthesis of both substances proceeds and vice versa to which their disintegration leads. Since lignocerylsphingosine (26) is found to be present in organs together with cerebrosides and sphingomyelin, it was thought possible that sphingomyelin is built from this ceramide by esterification with phosphorylcholine while cerebrosides may result from its combination with a monosaccharide in glucoside linkage. This suggestion (27) is expressed as a hypothesis in figure 1.

In order to estimate the amount of cerebrosides in organs and serum several methods are employed (28, 29, 30, 31). The principle of these methods is based on the sugar content of the cerebroside molecule. The sugar is split from the molecule by acid hydrolysis. The galactose is determined colorimetrically: the glucose by fermentation. Since the quantities of cerebrosides in normal organs are very small their extraction does not yield pure substances necessary for colorimetric determination.

The Gaucher cells, in contrast to the foam cells in xanthomatosis and in Niemann-Pick's disease, are very large and homogeneous. They are usually multinucleated and do not stain dark blue with Lorrain-Smith-Dietrich stain.

The adult form of Gaucher's disease occurs in families and singly. The onset of the disease is so insidious that the first symptoms may escape observation. An enlarged spleen may develop as the first sign or pain located in the skeletal system may, without visible enlargement of the spleen, constitute the first symptom of the disease. Patchy pigmentation of the face similar to that seen in cloasma uterinum may be seen in later stages. A wedge-shaped, darkly pigmented thickening of the conjunctiva on both sides of the pupils as well as linear

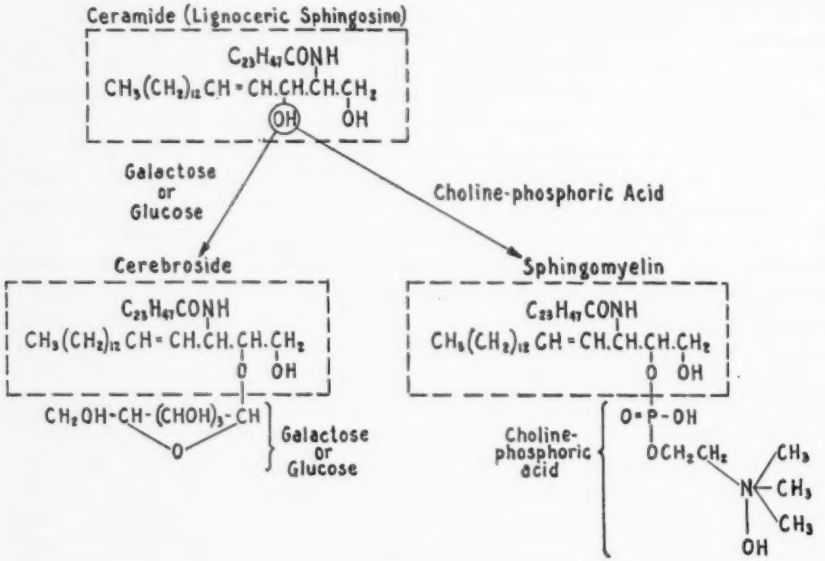


Fig. 17

pigmentation of the legs is not a constant symptom of the disease. The increase in the size of the liver and the lymph glands is observed subsequent to the enlargement of the spleen. The size of the spleen may become enormous and is always more impressive than the size of the enlarged liver. Severe pains of the bones occur in intervals and are accompanied by fever. In some patients the osseous involvement proceeds without pain and fever and is only discovered by systematic X-ray examination of the entire skeleton. The X-ray picture of the femur reveals in most cases a characteristic deformity. The waistline of the femur above the condyles disappears because the thinned cortex of the bone seems to be expanded and inflated in this area, probably as the result of the replacement of the marrow by Gaucher cells, giving the distal end of the femur the contours of an Erlenmeyer flask.

The diagnosis of Gaucher's disease is made by its clinical features and by the

X-ray pictures of the bones. It should be confirmed by a bone marrow biopsy showing the characteristic Gaucher cell. As already mentioned the chemical analysis of the blood serum reveals nothing characteristic. The morphological examination of the blood shows in some cases anemia, leukopenia and thrombocytopenia. These features of pancytopenia are usually found in cases in which the bone marrow is replaced by other tissues; in the disease under discussion the marrow is replaced by Gaucher cells.

The course of the adult form of Gaucher's disease depends on the age as well as on the speed at which the symptoms develop. The younger the patient the more rapid is the course of the disease. As a rule the course of the disease in adults is protracted and may last a life time. Tuberculosis is a frequent complication of Gaucher's disease.

In the so-called "infantile form" of Gaucher's disease the development differs, in that not only the organs of the lymph-hemopoietic system are involved but Gaucher cells develop in the brain, lungs, and most of the organs (32). The symptoms resulting from the involvement of the brain dominate the clinical picture. As a result of the extension of the disease to all organs the clinical features and the rapid progress of the disease are not unlike that observed in Niemann-Pick's disease. The finding of Gaucher cells in bone marrow biopsies determines the diagnosis.

D. Tay-Sachs Disease (*Amaurotic Idiocy, Juvenile Type*). Sachs (1) described in 1896 "A Family Form of Idiocy Generally Fatal Associated with Early Blindness." The ophthalmological features of these diseases had already been published in 1881, by the English ophthalmologist W. Tay (2). Since characteristic changes in the retina, characterized by a cherry red spot within a grayish green halo occupying the place of the macula and progressive mental deterioration occur in Niemann-Pick's disease as well as in Tay-Sachs' disease it was thought that both diseases are related in respect to their etiology. Thannhauser (3), however, showed that the sphingomyelin content of organs was not increased in Tay-Sachs' disease in contrast to the accumulation of sphingomyelin in Niemann-Pick's disease. Also the histological examination of the retina (4) revealed differences in the histological changes of the retina in both diseases. The chemical difference of Tay-Sachs' disease and Niemann's was finally established by E. Klenk, by the isolation of a new group of lipids from the brain of infants suffering from Tay-Sachs' disease. Klenk named this new group of lipids "gangliosides" (Substance X). The chemical properties of the gangliosides are discussed on page 287 (5, 6). The gangliosides are present in normal brain, but are increased in the brain of Tay-Sachs' disease replacing to some extent the cerebrosides (7).

In Tay-Sachs' disease the ganglioside content of the total solids of the brain is found to be 4–8 per cent, in contrast to 0.3 per cent in the normal brain. There is a question whether two types of amaurotic idiocy occur. The one type is called Tay-Sachs' disease, the other is designated the "juvenile type of amaurotic idiocy." In the latter type, Klenk found the ganglioside content of the brain only slightly increased (1.5 per cent). Slightly increased values of gangliosides are also found in Niemann-Pick's disease. It is however definitely established

that Niemann-Pick's disease is entirely distinct from Tay-Sachs' disease as the sphingomyelin content of all organs in Tay-Sachs' disease is normal.

E. Pfaundler-Hurler's Disease (Gargoylism). It is questionable whether changes in lipid metabolism are responsible for this rare syndrome. Up to the present there are no chemical findings reported which favor such an etiology of this disease (1, 2, 3, 4, 5).

SUPPLEMENT

Inositolphosphatides: J. Folch (Fed. Proc. **5,** 134 (1946)) succeeded in further purifying the inositolphosphatide fraction of brain. The complete acid hydrolysis of the product yielded inositol phosphoric acid, glycerol and fatty acids in ratios 1:2:1:1. In the intact phosphatide both phosphoric acid groups are esterified with inositol since a short acid hydrolysis resulted in the formation of inositol-m-diphosphoric acid ester.

The phosphatide contained 16% inositol. It accounts for the total amount of inositol present in the crude cephaline fraction. The nitrogen content of 0.6% is far below that required for the presence of one equivalent of base. It is not yet established whether the N containing group is a part of the phosphatide molecule or whether it is a contamination.

Cardiolipin: On saponification with alcoholic potassium hydroxide, cardiolipin yields an organic P ester which is very easily hydrolyzed by dilute mineral acids (W. C. Pangborn, Fed. Proc. **5,** 149 (1946)). The only hydrolysis products formed are glycerol and glycerophosphoric acid. Pangborn assumes, therefore, that cardiolipin represents a phosphatidic acid in which glycerophosphate is replaced by a complex glyceryl-glycerophosphate.

Glycerylphosphorylcholine: E. Kahane and J. Levy (Compt. rend. 219, 431 (1944)) found in rat intestines a lecithinase B which hydrolyses lecithin to fatty acids and to a watersoluble choline compound which contained glycerophosphate. The properties of the crude fraction obtained by Kahane and Levy are similar to those of the pure glycerylphosphorylcholine isolated from pancreas by Schmidt, Hershman and Thannhauser. Kahane and Levy found that their fraction has no pharmacological effects.

Independently G. Schmidt, L. Hecht and S. J. Thannhauser (J. Biol. Chem., in print) found that lecithin disappears rapidly during the autolysis of minced rat intestines. Simultaneously, free choline and glycerylphosphorylcholine appear in amounts corresponding to those of the hydrolyzed lecithin.

E. J. King and W. Aloisi (Biochem. J. **39,** 470 (1945)) isolated from beef pancreas a watersoluble choline ester of glycerophosphate with the ratios choline: glycerophosphate = 2:1.

Determination of phospholipids: G. Blix (Biochem. Zs. **305,** 129 (1940); J. Biol. Chem. **139,** 471 (1941)) determined colamine in hydrolysates of phospholipids by vacuum distillation under 10 mm. Hg pressure at 75–80° and following acidimetric titration.

Determination of cerebrosides: Brückner has recently applied the orcinol test to the separate determination of glucose and galactose in mixtures of both sugars.

He described on this basis a procedure for the separate determination of galactose and glucose containing cerebrosides (Ztschr. physiol. Chem. **275,** 73 1942)).

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III. *Lipidoses*

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